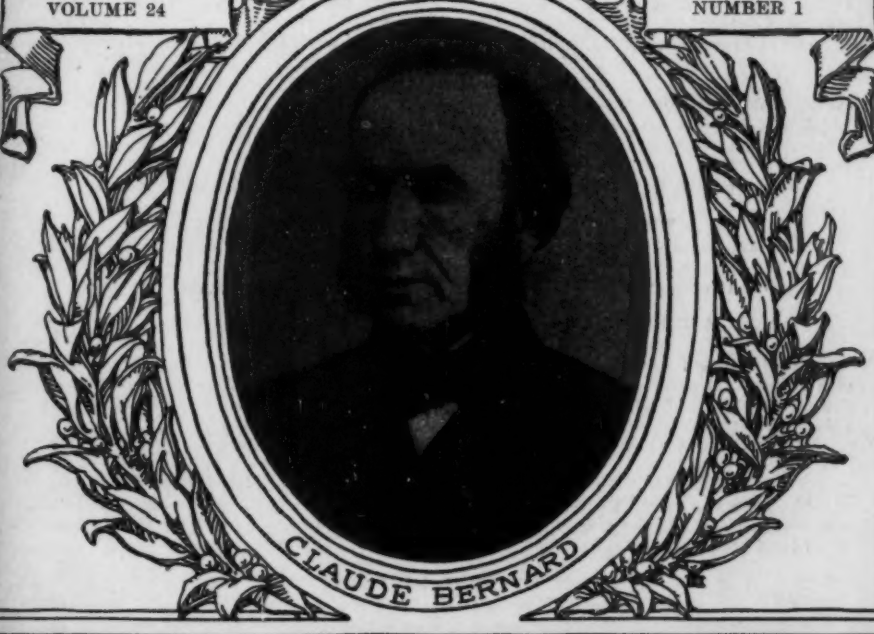
JULY 10, 1942

THE JOURNAL OF NUTRITION

VOLUME 24 NUMBER 1



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Official Organ of the American Institute of Nutrition
PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PA.
Price, $5.00 per volume. Domestic; $5.50 per volume, Foreign

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THE MOBILIZATION BY ALCOHOLS OF VITAMIN A FROM ITS STORES IN THE TISSUES

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(Received for publication February 19, 1942)

INTRODUCTION

The experiments to be reported in this paper are concerned with the increase in concentration of vitamin A of the serum of dogs following the administration of various alcohols. The experimental conditions were so arranged that the increase was not due to increased absorption of the vitamin from the intestinal tract. We shall attempt to show that the increase was due to liberation of the vitamin from stores in the tissues.

The experiments were undertaken to elucidate the manner in which vitamin A may be liberated from its stores in normal and in pathological conditions. Clausen and McCoord ('38) had observed that the concentration of vitamin A of the plasma increases above the normal level several days after the fall in temperature in scarlet fever and in pneumonia. A similar rise in vitamin A concentration of the plasma was observed 1 or 2 days after artificial hyperthermia; this rise may, of course, be due to a decreased rate of deposition in the stores of vitamin A absorbed from the gastrointestinal tract. During convalescence from acute infectious jaundice an even greater temporary rise in level of plasma vitamin A is observed; this rise can most readily be ascribed to liver damage, which results in failure of rapid storage of vitamin A absorbed from the gastrointestinal tract into the blood or lymph, and not to liberation of the vita-

THE JOURNAL OF NUTRITION, VOL. 24, NO. 1
JULY, 1942
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min from its stores. Somewhat more definite evidence for liberation of vitamin A from tissue stores was found in two children dying of chronic nephritis: Unusually high concentrations were found in the plasma, and very low values were found in the liver. In experiments with rats (which had received a single massive dose of vitamin A and thereafter had received a vitamin A free diet), McCoord ('39) observed that those animals which succumbed to infection with B-paratyphosus muris, had much more vitamin A in the adrenals than did controls; whereas, the vitamin A content of the liver and lung had decreased in the infected animals. After rats had received large daily doses of thyroxin for several weeks the vitamin A content of the livers decreased, while that of the adrenals increased.

Solyanikova ('38) claimed that doses of vitamin D produced first an increase in the vitamin A content of the blood of rats and a decrease in the vitamin A content of the liver and suprarenal capsule. Later, the vitamin A content of the blood and liver decreased while that of the suprarenal capsule increased.

Young and Wald ('40) showed that, in rabbits, the concentration of vitamin A in the blood was increased by the extirpation of a lobe of the liver, by electrical stimulation of the splanchnic nerve, and by the intravenous injection of adrenaline.

Recently Clausen et al. ('40 a) showed that vitamin A could be mobilized from its stores in the tissues by ethyl alcohol. Previously, attempts were made without marked success to mobilize vitamin A by various drugs and experimental procedures. We have studied the effect of injection of adrenaline, histamine, insulin, mecholyl chloride, exsanguination, inhalations of chloroform and of ether, and operative trauma. Repeated bleeding of rabbits gave some evidence of mobilization of vitamin A, for, although considerable amounts of blood were withdrawn, the total quantity of the vitamin in the circulating blood remained almost constant. Prolonged ether anesthesia at times produced a slight rise in the vitamin A concentration of the serum. However, none of these agents

produced an effect comparable to that of ethyl alcohol. Using dogs as subjects, these authors showed that there was a prompt increase in the vitamin A concentration of the serum following the administration of the alcohol.

Pett has devised a method for measuring the rate of visual adaptation of man to dim light. He reported ('40) that this rate was unusually rapid on the day following the ingestion of alcohol, and ascribed the phenomenon to liberation of vitamin A from its stores. Clausen and his associates ('41) then showed that there was an increase in the vitamin A concentration of the serum of man following the ingestion of ethyl alcohol.

On the other hand, Colson ('40) found that tests given soon after the consumption of alcohol by normal individuals showed no significant change in visual acuity, visual fields, color vision and dark adaptation.

In the present paper we report the results of studies of the effects of various alcohols upon the mobilization of vitamin A from its stores in the tissues of the dog.

METHODS

The methods for the extraction of vitamin A from serum and tissues are described in detail elsewhere (McCoord and Luce-Clausen, '34; Clausen and McCoord, '38).

The vitamin A content of the petroleum ether extracts was determined by means of the Evelyn photoelectric colorimeter according to the technic first suggested by May (Dann and Evelyn, '38), except that the residue containing the vitamin A was dissolved in 0.5 ml. of chloroform, and 10 ml. of the antimony trichloride reagent was added. For the sake of brevity, we shall use the abbreviation E. P. U. for the Evelyn Photoelectric Unit of vitamin A. One E. P. U. is equal to about 3 International Units of vitamin A. The method for the determination of the free and esterified forms of vitamin A has been described briefly (Clausen et al., '40 b); a more detailed report will be published later.

The vitamin A concentration of the serum in normal dogs following the administration of various alcohols

Twelve dogs were used as subjects in this study, a total of twenty-three tests being carried out. The animals were maintained on a diet of commercial dog food [1] with the addition of a small piece of lean meat each day. In all tests, the alcohol was administered in a single dose. In eleven tests the dogs received ethyl alcohol by stomach tube as a 20% aqueous solution by volume of 95% ethyl alcohol, and in five tests by vein as a 30% solution by volume of 95% ethyl alcohol in isotonic salt solution. Three subjects received methyl alcohol by vein as a 30% solution by volume in isotonic salt solution. By vein dog 38-309 received 10 ml. of propyl alcohol diluted with 10 ml. of the salt solution; dog 39-11, 10 ml. of isopropyl alcohol diluted with 20 ml. of the salt solution; and dog 39-191, 5 ml. of butyl alcohol diluted with 65 ml. of the salt solution. The protocols of six of the experiments are given in table 1. In all the dogs of our series, there was a prompt increase in the vitamin A concentration of the serum following the administration of the alcohol except in the case of the animal which received only 5 ml. of butyl alcohol. The amounts of alcohol used in these experiments varied between 5 and 60 ml. Six of the subjects were given more than one test, but the effect of the alcohol did not appear to diminish with a repetition of the test.

While the dogs receiving the larger doses of ethyl alcohol were very drowsy for some hours, neither ethyl nor methyl alcohol appeared to be injurious to the animals in the doses given. Propyl, isopropyl and butyl alcohols were given by vein in doses of 5 to 10 ml. well diluted with the salt solution; they appeared to be quite intoxicating. The dogs barked, struggled and seemed much excited and then became very drowsy. Their blood showed marked hemolysis and in one case hemoglobinuria was observed. However, the animals seemed to recover rapidly.

[1] Purina Dog Chow.

The coefficient of correlation between the number of milliliters of 95% ethyl alcohol administered and the greatest increase in Evelyn Photoelectric Units of vitamin A per 100 ml. of serum obtained within the 48 hours was 0.447.

We have found that the diet used in the experiments caused little variation in vitamin A concentration of the serum of dogs during the day, or even in the course of several days. To rule out the effect of the absorption of vitamin A from the gastrointestinal tract, food was withheld from the dogs from 18 to 48 hours previous to and during, the experiments.

Biopsies of the liver were obtained in nine of the dogs receiving 95% ethyl alcohol. The coefficient of correlation between the concentration of vitamin A per 100 gm. of liver and the greatest increase in Evelyn Photoelectric Units of vitamin A per 100 ml. of serum obtained within the 48 hours was 0.280.

TABLE 1

The vitamin A concentration of serum in normal dogs following the administration of various alcohols.

Dog:						
No.	39–11	38–314	38–309	38–309	39–11	39–191
Body weight (kg.)	17	18	21	21	17	16
Treatment:						
Alcohol	Ethyl 95%	Ethyl 95%	Methyl	Propyl	Isopyl	Butyl
Amount	60 ml.	20 ml.	20 ml.	10 ml.	10 ml.	5 ml.
How given	Stomach tube	Vein	Vein	Vein	Vein	Vein
Hours of fasting before test:	48	18	24	24	24	24
Serum: Vit. A per 100 ml. (E.P.U.[1]):						
Before test	569	416	638	488	202	140
1½ hours after test	...	...	702	781	255	140
3 hours after test	1323	...	1172	868	255	103
6 hours after test	...	862	....	868	...	129
24 hours after test	914	757	2230	908	206	...
48 hours after test	859	...	2041	830	...	...
Liver: Vit. A per 100 gm. (E.P.U.[1]):	8,134	27,196	106,314	106,314	8,134	5,975

[1] Evelyn photoelectric unit.

However, the amount of ethyl alcohol given varied between 16 and 60 ml. It is probable that if all the dogs had received an amount of alcohol proportional to their body weights, a better correlation would have been obtained. In two dogs with fistulas of the thoracic duct, the vitamin A concentration of the lymph did not increase after the administration of the alcohol, but a rise in the vitamin A of the serum did occur.

To ascertain from what tissues of the dog vitamin A could be mobilized, three normal animals were sacrificed and the vitamin A concentration and content of twenty-four tissues of each dog were determined. The detailed results of these analyses are to be published in another communication. It was found that vitamin A is widely distributed in the tissues of the dog, both the concentration and total amount of the vitamin being relatively great in the liver, the kidneys, the retroperitoneal and the subcutaneous fat. The high vitamin A content of the kidney may be related to the fact that the vitamin was frequently found in the urine of normal dogs. On the other hand, the gastrointestinal tract, with its contents, contained a very small amount of vitamin A, and can therefore be definitely eliminated as the source of the mobilized vitamin.

Attempts were made to show that the vitamin was liberated directly from the liver, by injecting the alcohol into the portal vein and then analyzing simultaneously the blood from the hepatic vein and the leg vein for some hours thereafter. The results were rather conflicting, so that no definite conclusions could be established.

The administration of ethyl acetate, acetone, paraldehyde, acetaldehyde, acetic acid and hydrochloric acid was not followed by a rise in the vitamin A concentration of the serum.

The effect of the daily administration of ethyl alcohol

Daily injections of ethyl alcohol were given to three normal dogs, the procedure being as follows: Each morning for 8 days blood specimens were taken, and the dogs then received by vein, 20 ml. of 95% ethyl alcohol diluted with 40 ml. of sterile isotonic salt solution. In the afternoon at about four o'clock,

blood specimens were again obtained and the dogs were given the usual diet. Each dose of alcohol was usually followed by a rise in the vitamin A concentration of the serum. Table 2 gives the protocol of one of these dogs, no. 38-314. However, by the ninth day the tissues surrounding the veins of the animals were so edematous that it was decided to inject the alcohol into the peritoneal cavity. At the same time the dose

TABLE 2

Effect of the daily administration of ethyl alcohol on the vitamin A concentration of the serum of a normal dog.

DATE	ALCOHOL	TIME	E.P.U.[1] VITAMIN A PER 100 ML. SERUM	TIME	E.P.U. VITAMIN A PER 100 ML. SERUM
	ml.	*A.M.*		*P.M.*	
3–18–40	20 — by vein	10	416	4	862
3–19–40	20 — by vein	10	757	7	1055
3–20–40	20 — by vein	10	694	4	1080
3–21–40	20 — by vein	10	932	4	1068
3–22–40	20 — by vein	9	872	4	1367
3–23–40	20 — by vein	9	951	4	1232
3–24–40	20 — by vein	9	1131	4	1244
3–25–40	20 — by vein	9	958	4	1203
3–26–40	40 — intraperitoneal	9	818	4	1491
3–27–40	40 — intraperitoneal	9	1046	4	1128
3–28–40	20 — intraperitoneal	9	923	4	327
3–29–40	20 — intraperitoneal	9	1818	4	1528
3–30–40	20 — intraperitoneal	9	1147	4	1115
3–31–40	20 — intraperitoneal	12 noon	903	6	1188
4–1–40	20 — intraperitoneal	9	942	2	1199

[1] Evelyn photoelectric unit.

was increased to 40 ml. of alcohol diluted with 80 ml. of isotonic salt solution. This amount of alcohol proved to be too much, for the dogs slept all of the time and refused to eat. The former dose of 20 ml. of alcohol and 40 ml. of the salt solution was therefore resumed and administered for 5 days. The alcohol was as effective in mobilizing the vitamin A when injected into the peritoneal cavity as when injected by vein. Each dog received fifteen daily injections, a total of 340 ml. of 95% alcohol.

Biopsies of the livers of these dogs and of a control dog were obtained at the beginning and at the end of this experiment. During this period, the concentration of vitamin A in the liver of the control dog, no. 38-272, increased from 31,630 to 35,575 E. P. U. per 100 gm. of tissue, while that of experimental dog no. 38-314 increased from 27,196 to 29,921 units. On the other hand, the concentrations of vitamin A in the livers of the experimental dogs, no. 39-11 and 39-219, decreased from 8,134 to 2,791 and from 1,619 to 974 units per 100 gm., respectively. Unfortunately, we have no accurate record of the intake of vitamin A by the dogs during the period. These changes were not due to differences in the moisture content of the tissues analyzed. Nor is it probable that they were due to errors in sampling, since we have found that specimens taken at the same time from different parts of the periphery of the liver have approximately the same vitamin A concentration. Microscopic sections of the biopsy specimens of these livers were also prepared. Dr. W. B. Hawkins of the Department of Pathology, who very kindly examined the sections, found that there was no significant alteration in the cells of the livers after daily injections of ethyl alcohol.

The urine of these dogs contained vitamin A, but the quantity present was not increased by the administration of the alcohol.

The effect of alcohol on the absorption of vitamin A in dogs

When normal dogs receive a large dose of vitamin A by mouth, there is a marked increase in the vitamin A concentration of their serum, the highest concentration usually being reached within 12 hours. It seemed important to determine whether alcohol, administered at the same time, would influence this effect. Three normal dogs received in a capsule by mouth 1800 E. P. U. of vitamin A per kilogram of body weight after a fasting blood specimen had been taken. The vitamin A concentration of the serum was determined at intervals during the next 30 hours. After a few days had elapsed, the dogs received by stomach tube 40 ml. of 95% ethyl alcohol di-

luted with isotonic salt solution, and the rise of vitamin A in their serum was determined as in the preceding experiments. A few days later the tests were repeated, the vitamin A and alcohol being administered at the same time.

The results obtained for two of the dogs are presented in table 3. In the case of dog 39-191, a much greater effect was obtained when the vitamin A and alcohol were given together than when either was given alone. This effect, however, was

TABLE 3

The effect of ethyl alcohol on the absorption of vitamin A in dogs.

No. of dog	39–191			39–11		
Body weight (kg.)	16			17		
Date	5–22–40	5–27–40	6–3–40	5–27–40	5–22–40	6–3–40
Treatment	28,800 E.P.U. vit. A by mouth	40 ml. 95% ethyl alcohol by stomach tube	28,800 E.P.U. vit. A by mouth + 40 ml. 95% ethyl alcohol by stomach tube	30,600 E.P.U. vit. A by mouth	40 ml. 95% ethyl alcohol by stomach tube	30,600 E.P.U. vit. A by mouth + 40 ml. 95% ethyl alcohol by stomach tube
Serum: Vit. A per 100 ml. (E.P.U.[1])						
Before test	276	255	230	142	224	188
4 hours after test	569	447	671	438	428	410
9 hours after test	717	460	748	630	478	515
12 hours after test	856	608	1210	621	447	515
24 hours after test	830	716	1493	459	272	468
30 hours after test	793	584	1414	...	...	476

[1] Evelyn photoelectric unit.

not observed in the case of the other two dogs. It may be that, in some subjects, alcohol retards the absorption of the vitamin by decreasing the motility of the intestine, or that it causes rapid elimination of the vitamin by way of the intestinal tract. Some of our dogs were observed to defecate while alcohol was being injected by vein.

The determination of the free and esterified forms of vitamin A of serum of dogs after administraton of alcohol

The concentrations of free and of esterified vitamin A in the serum of three dogs which had received methyl alcohol were

determined. It was found that the rise in vitamin A was due almost wholly to the rise of the esterified form while the concentration of the free form remained practically unchanged. Table 4 gives the protocol of one experiment. We have not completed similar analyses of the serum of dogs treated with other alcohols.

Since the evidence available indicated that the mobilized vitamin A was derived from the stores in the body, the form in which the vitamin was present in these stores was investigated

TABLE 4

The concentrations of free and esterified vitamin A in dog serum following the administration of methyl alcohol.

Dog 38–309.	20 ml. methyl alcohol by vein.		Wt. = 21 kg.
TIME	E.P.U.[1] VITAMIN A PER 100 ML. OF SERUM		
	Free	Esterified	Total
hours			
Before alcohol	42	587	629
1½ after alcohol	67	687	754
4 after alcohol	23	1117	1140
19 after alcohol	27	2462	2489
26 after alcohol	21	2782	2803
44 after alcohol	42	2300	2342
68 after alcohol	50	1311	1361

[1] Evelyn photoelectric unit.

in two normal dogs. The dogs were sacrificed and samples of liver, kidneys, adrenals, retroperitoneal fat, and subcutaneous fat, taken for analyses. The results are summarized in table 5. It was found that in all of these tissues, with the exception of the kidneys of dog 39-11, the vitamin was present mainly as the ester.

The concentrations of the free and esterified forms of vitamin A in the urine of three dogs were also determined. As is evident from table 5, both forms are present and either may predominate.

TABLE 5

Free and esterified vitamin A in the tissues and urine of dogs.

NO. OF DOG		E.P.U.[1] VITAMIN A PER 100 GM. OF SUBSTANCE		
		Free	Esterified	Total
39–11	Liver	123	1537	1660
	Kidneys	589	567	1156
	Adrenals	99	2725	2824
	Subcutaneous fat	149	285	434
	Abdominal fat	100	3884	3984
39–168	Liver	260	7524	7784
	Kidneys	311	1796	2107
	Adrenals	34	1541	1575
	Subcutaneous fat	45	1599	1644
	Abdominal fat	87	1143	1230
	Urine	20	6	26
40–80	Urine	77	2	79
40–104	Urine	4	13	17

[1] Evelyn photoelectric unit.

DISCUSSION OF RESULTS

Our experiments clearly show that when dogs received methyl, ethyl, propyl or isopropyl alcohols either by mouth, vein, or injection into the peritoneal cavity, there was a prompt increase in the concentration of vitamin A in their serum. This increase was not due to increased absorption of the vitamin from the gastrointestinal tract, for the following reasons: (1) Dogs which had been fasting for 48 hours showed no decrease in the vitamin A mobilized; (2) the diets used in these experiments caused little variation in the vitamin A concentration of the serum during the course of the day, or even in the course of several days; (3) the gastrointestinal tracts of dogs receiving the diet contained little vitamin A; (4) in two dogs with fistulas of the thoracic duct, the vitamin A concentration of the lymph did not increase after the administration of the alcohol, but a rise in the vitamin A of the serum did occur.

We concluded that the mobilized vitamin A must have been derived from the liver and extrahepatic stores, for the follow-

ing reasons: (1) Almost all of the vitamin A present in these stores was in the form of the ester; (2) almost all of the mobilized vitamin A in the serum was in the form of the ester; (3) in two dogs which received daily injections of ethyl alcohol, there was a marked decrease in the vitamin A of their livers during the course of the experiment.

The increase of vitamin A in the serum cannot be ascribed to the conversion of carotene to the vitamin under the influence of alcohol, for carotene is present in the tissues of the dog in only minute amounts.

The presence of both free and esterified vitamin A in the tissues may lead to a better understanding of the metabolism of vitamin A by the body. Clausen et al. (40 c) showed that nearly all the vitamin A in the serum and urine of children was in the free form. Recently we determined the form of vitamin A in many tissues of man, cat, rabbit, guinea pig, chicken, gopher and monkey. A small amount of the free vitamin was always present, but when the concentration of the vitamin was relatively large, the greater part was in the ester form. It therefore appears that vitamin A is stored and transported mainly as the ester. The studies reported in this paper do not warrant conclusions being drawn regarding the function of the small amount of the free vitamin present in the tissues.

CONCLUSIONS

After the administration of methyl, ethyl, propyl and isopropyl alcohols to dogs, there was a marked increase in the vitamin A concentration of the serum, the maximum rise usually being evident within 24 hours. The mode of administration of the alcohol, whether by mouth, vein, or injection into the peritoneal cavity, did not affect the results. It was shown that the effect was not due to increased absorption of the vitamin from the gastrointestinal tract. The vitamin A was mobilized from its storage places in the body, such as the liver and fat depots. Almost all of the mobilized vitamin A and the greater part of the vitamin present in the tissues of the dogs was found to be

in the form of the ester. There was, however, a small amount of free vitamin A present. It is therefore suggested that vitamin A must be in the free form in order to be utilized by the tissues, and that it is stored as the ester. In dogs which received daily doses of ethyl alcohol, the rise in the vitamin A concentration of the serum occurred each day. During the course of this experiment, the concentration of vitamin A in the liver decreased in the cases of two dogs whose original stores were low, but increased in the case of a third dog with initially higher stores.

We wish to express our thanks to Dr. Carl Nielsen of the Abbott Laboratories for supplying us with the fish liver oil used in these experiments, and to Mr. W. H. Richardson for his valuable assistance in carrying out these studies.

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CYSTIC PITUITARY IN YOUNG CATTLE WITH VITAMIN A DEFICIENCY [1]

LOUIS L. MADSEN, SAMUEL R. HALL AND H. T. CONVERSE

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TWO PLATES (FIVE FIGURES)

(Received for publication January 12, 1942)

In connection with both pathological studies on tissues from cattle on deficient diets and certain physiological and histological studies on the pituitary gland, routine examinations have been made on this gland from young beef and dairy cattle either suffering from vitamin A deficiency or having a previous history of vitamin A depletion. In the glands examined a large cyst containing clear serum-like fluid has been found in most cases [2]. The cyst usually occupies space in or between the posterior and anterior lobes, often causing considerable pressure atrophy of the surrounding glandular parenchyma. In some cases the cyst appears to have formed in the residual lumen but in others it appears to be within the posterior lobe only, causing distortion of the entire gland.

References to this condition in cattle are limited. Moore ('39) briefly noted cysts in the pituitary of vitamin A deficient calves and suggests their presence as "further evidence of disturbance in the region of the cranial cavity" during this

[1] This research was supported in part by an appropriation from Bankhead-Jones funds (Bankhead-Jones Act of Congress, June 29, 1935).

[2] Cases of cystic pituitary were shown in the United States Department of Agriculture educational exhibit given in connection with the 77th Annual Meeting of the American Veterinary Medical Association, Washington, D. C., August 26–30, 1940.

deficiency. Sutton, Krauss and Hansard ('40) have also reported "accumulation of fluid in the cleft between the anterior and posterior lobes of the pituitary" in young bulls fed a vitamin A deficient ration for about 1 year. These latter workers reported an increase in alpha cells in the anterior lobe of such a pituitary and on assay this portion of the gland showed increased gonadotropic hormone content. This was believed to indicate compensatory activity in the pituitary resulting from injury to the testes caused by the deficiency of vitamin A. This view is consistent with the earlier findings of Sutton and Brief ('39) in vitamin A deficient rats in which gonadotropic activity of the pituitary was found to be higher than normal and the cellular changes in the gland were interpreted to be similar to those found after castration. The mechanism responsible for increased gonadotropin in the anterior lobe of vitamin A deficient bulls is difficult to explain on a basis of testis injury simulating castration since Bates, Riddle and Lahr ('35) using the immature male pigeon as the test animal found steers to be less potent in this factor than bulls. One of us (S. R. H.) and Dr. J. T. Bradbury have also recently found the bull to be more potent in this factor as indicated by the rabbit ovulation test.

None of the previous reports has emphasized either the finding of pressure atrophy or evidence of other degenerative changes in the pituitary of cattle suffering from vitamin A deficiency; such phenomena are described in the present work.

EXPERIMENTAL OBSERVATIONS

During studies on the gonadotropic and lactogenic hormones of the anterior lobe of the pituitary more than 10,000 pituitaries have been collected from apparently normal cattle slaughtered at the Beltsville Research Center and at a meat packing plant in Baltimore, Maryland. In this group only four glands, and possibly a fifth, have been encountered that were definitely cystic. The rare occurrence of cystic pituitary glands in normal cattle is in marked contrast to the large number

found in a group of young cattle that developed varying degrees of vitamin A deficiency while on experimental rations.

In a series of fifteen vitamin A deficient animals of beef and dairy ancestry varying in age from the day of birth to 787 days of age, thirteen were found to have cystic pituitary glands. The beef animals consisted of one Shorthorn (cow 234), and a group of Shorthorn-Hereford crossbreds produced in connection with a study of the vitamin A requirement for reproduction. The dairy animals had been used in a study on the vitamin A requirement for growth and health of young animals. In this group one was a Jersey (steer 310-B) and the others were Holsteins. These cases are summarized in table 1.

The cystic pituitary of cow 234 was noted in a previous publication by Davis and Madsen ('41). This cow was depleted of vitamin A for a period of 125 days beginning at 23 days of age. Marked symptoms of deficiency appeared, including papilledema, low blood carotene, and low blood vitamin A, convulsions, diarrhea, night blindness and slow growth. The animal was then given enough high-grade alfalfa leaf meal of known carotene content to supply 120 μg. of carotene per kilogram of body weight per day. Within a short time the deficiency symptoms disappeared and apparently normal health was regained; however, the animal failed to grow normally. She conceived at 18 months of age and aborted during the eighth month of pregnancy. She was slaughtered 4 days after aborting and at this time the chief autopsy findings were small under-developed ovaries and a cystic pituitary gland. No specific cause for abortion was established. The pituitary of this cow (figs. 1, 2, and 3) is of special interest since the effects of the cystic condition were still apparent after a long period of adequate carotene intake. A considerable proportion of the functional anterior pituitary was replaced by the cyst and a large part of the remaining glandular parenchyma was replaced by fibrous connective tissue in which were scattered small groups of anterior lobe cells and smaller cysts. Many

TABLE 1

Occurrence of pituitary cysts in young beef and dairy cattle receiving an insufficient amount of vitamin A or carotene.

ANIMAL NO.	SOURCE AND AMOUNT OF VITAMIN A AND CAROTENE FED			AGE AT SLAUGHTER	DEFICIENCY SYMPTOMS	PITUITARY CYST
	Whole milk	Yellow carrots [1]	Carotene in oil [2]			
Beef cattle	*days*	*grams daily*	*μg./kg. body wt.*	*days*		
Cow 234	23			787	In calfhood only	Large
Heifer 166-1	103			103	Papilledema after 30 days	Very large
Heifer 78-1	44			44	Born blind; convulsions	Very large
Heifer 173-1	87			87	Born blind; convulsions	Very large
Bull 173-2	31			31	Born blind; convulsions	Very large
Heifer 29-1	0			0	Died shortly after birth	Small
Bull 33-1	0			0	Died shortly after birth	None
Dairy cattle						
Steer P-2	98 [3]			282	Blind about 10 days	Very large
Steer 310-B	30	60 after 30 days		180	Blind about 17 days	Very large [4]
Steer P-16	4	60 to 7 months		540	Blind right eye at 254 days; both at slaughter	Very large
Steer P-21	4	60 to 7 months	29 after 7 months	540	Papilledema at 210 days	Large
Steer P-13	4	240 to 7 months	29 after 7 months	540	Night blind; occasional convulsions	Medium
Steer P-22	4	240 to 7 months		540	Blind several days	Very large
Steer 121-B	4		40	540	Blind left eye at 180 days; both at slaughter	Medium
Steer 144-B	4	1500 after 1 year	145 1st month 116 2nd month 58 3rd month 29 4th to 12th mo.	480	Suddenly blind at 313 days	None

[1] The carrots were grated and fed in milk for the first 30 to 60 days.

[2] The small amount of carotene furnished by timothy hay was deducted after the animals were 7 months of age and the balance was fed in oil solution.

[3] Whole milk was fed to 71 days and from 72 to 98 days equal parts of skimmed milk and whole milk were fed, after which only skimmed milk was given.

[4] Cyst contained 0.75 ml. of serum-like fluid.

of the remaining glandular cells had pycnotic nuclei and appeared non-functional.

Heifer 166-1 was apparently normal at birth but developed symptoms of vitamin A deficiency at about 30 days of age and died 73 days later. Carotene in oil solution failed to help this animal when the deficiency symptoms were well established. The pituitary of this calf (fig. 4) had a very large posterior lobe cyst which nearly replaced this lobe and caused considerable compression of the anterior lobe. The mother of this calf received 60 μg. of carotene in alfalfa leaf meal per kilogram of body weight daily during gestation and lactation. Several other calves born to cows on the same level of carotene intake have remained apparently normal.

The other beef calves were from cows receiving 30 to 45 μg. of carotene per kilogram of body weight. Symptoms of vitamin A deficiency were evident in these calves at birth and all of them had cystic pituitaries except bull 33-1 which died within a few minutes after birth.

The dairy animals examined in this study were all born to cows receiving sufficient carotene for normal reproduction. The calves were castrated at 60 days of age. All of them except 310-B and P-2 were given skimmed milk from the fourth day to either 6 or 7 months of age, together with a grain mixture having the following percentage composition: whole white corn meal, 30; wheat bran, 20; soybean meal, 25; and linseed meal, 25. Steers 310-B and P-2 were given considerably more whole milk and their grain mixture contained yellow corn meal in place of white corn meal. All of them were given liberal amounts of late cut low-carotene U. S. no. 3 timothy hay.

All of the dairy animals developed symptoms of vitamin A deficiency (table 1). Steers 310-B and P-16 received the least carotene and had very large pituitary cysts. Steers P-13 and P-22 received apparently adequate amounts of carotene up to 7 months of age, but steer P-22 still developed a cystic pituitary when the carotene supplement was discontinued, and steer P-13 when an inadequate amount of carotene was given.

Forty micrograms of carotene in oil per kilogram of body weight daily failed to protect steer 121-B from vitamin A deficiency. Blindness was noted in the left eye when the animal was about 6 months of age, and it was blind in both eyes at time of slaughter. At autopsy the left optic nerve was found to be completely constricted in the region of the optic foramen, and was smaller than normal throughout its entire length. The right optic nerve was only slightly constricted. The cyst in the pituitary of this animal was not large and histologically, no evidence of injury to the glandular parenchyma was seen.

Steer 144-B finally became blind from vitamin A deficiency, but it had an apparently normal pituitary at autopsy. This animal received large amounts of carotene early in life. After 12 months of age 1.5 kg. of carrots were added to the diet but no improvement in vision resulted.

DISCUSSION

The cases presented are consistent since they had either vitamin A deficiency at death or a previous history of vitamin A depletion, and all had cystic pituitary glands except steer 144-B and calf 33-1. Steer 144-B received more carotene than any of the other animals and from past experience one would have expected calf 33-1 to develop a cystic pituitary gland if it had survived. All of the animals, except the two calves which died shortly after birth, were known to have had visual defects and other symptoms confirming vitamin A deficiency. The high incidence of cystic pituitary glands in young cattle in which this deficiency has been experimentally produced suggests that this is a part of the pathology of vitamin A deficiency.

In the case of cow 234, the cyst persisted with little or no evidence of regeneration of functional anterior lobe in spite of a later adequate carotene intake. Steer 121-B shows that when the cyst of the pituitary is not large, histological evidence of injury to the surrounding glandular portions is lacking. When the cyst is large, however, enough of the glandular parenchyma is replaced or constricted to suggest partial

hypophysectomy. Results with steers P-13 and P-22 indicate that a cystic pituitary may develop in young cattle that are depleted after having received adequate amounts of carotene up to 7 months of age.

Moore ('39), and Moore and Sykes ('40) have shown that blindness in young bovines is associated with constriction of the optic nerve due to constriction of the optic foramen which in turn is probably due to increased intracranial pressure. Wolbach and Bessey ('40) have pointed out that a vitamin A deficiency in young animals (rats, guinea pigs, dogs), if established early, causes injury to the central nervous system. These authors suggest that this is due to growth of the nervous system not retarded by vitamin A deficiency, while the growth of bone, particularly endochondral growth of the bony enclosure of the central nervous system, is retarded. This results in mechanical injury to the brain, spinal cord, and nerve roots due to overcrowding.

It is highly probable that injury to the pituitary gland in calves results from the mechanism responsible for injury to both the optic nerve tracts and other parts of the central nervous system. Additional observations are needed to determine the relationship of age to the development of this condition, its effect on vital functions, and the effect of a period of deficiency in the calf on the subsequent usefulness of the animal.

SUMMARY

Cases of cystic pituitary glands have been found in young beef and dairy cattle either suffering from vitamin A deficiency or with a history of early severe vitamin A depletion. The cysts occurred either in the residual lumen or within the posterior lobe, often causing compression of the gland and injury to the glandular parenchyma. No evidence of repair in a cystic pituitary was found in an animal that was vitamin A deficient early in life but later fed adequate amounts of carotene, suggesting that the injury of the gland may be permanent.

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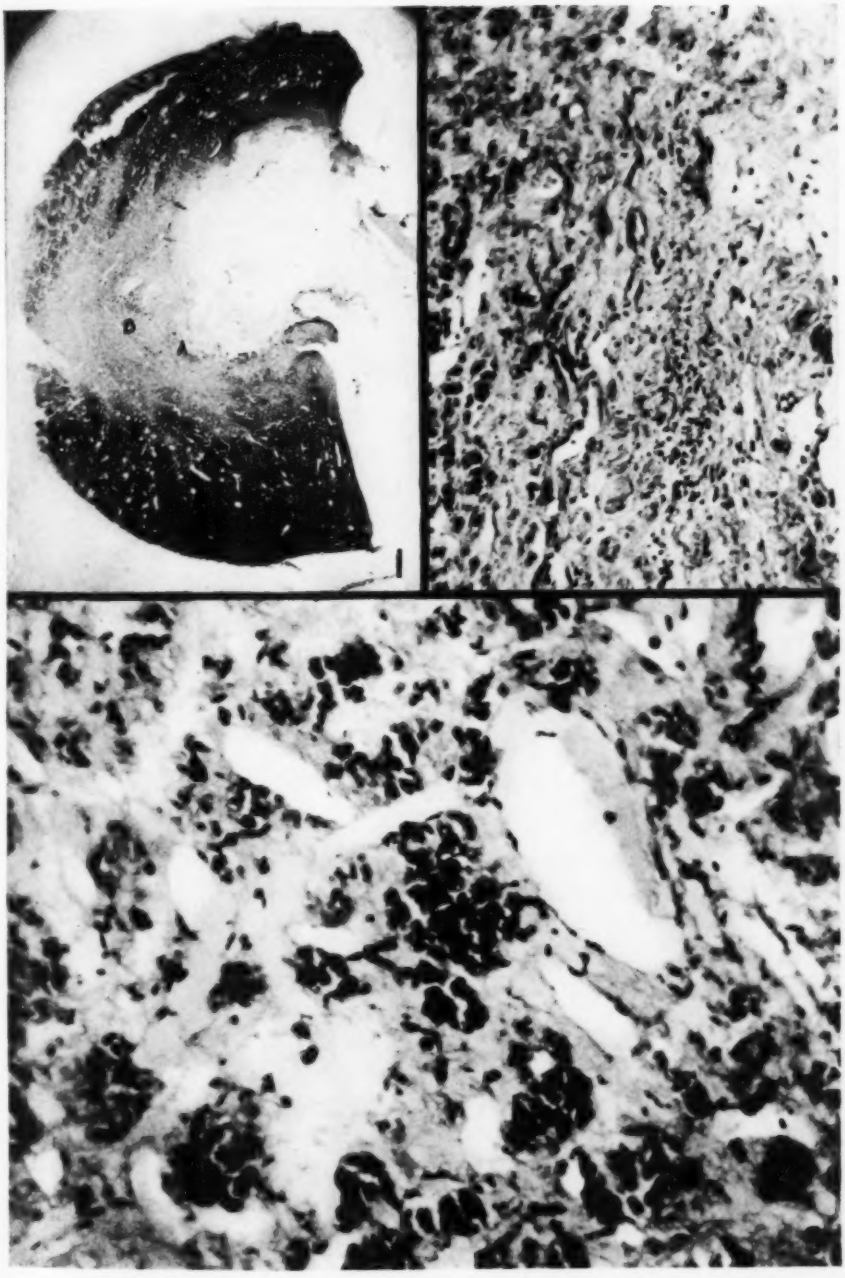
PLATE 1

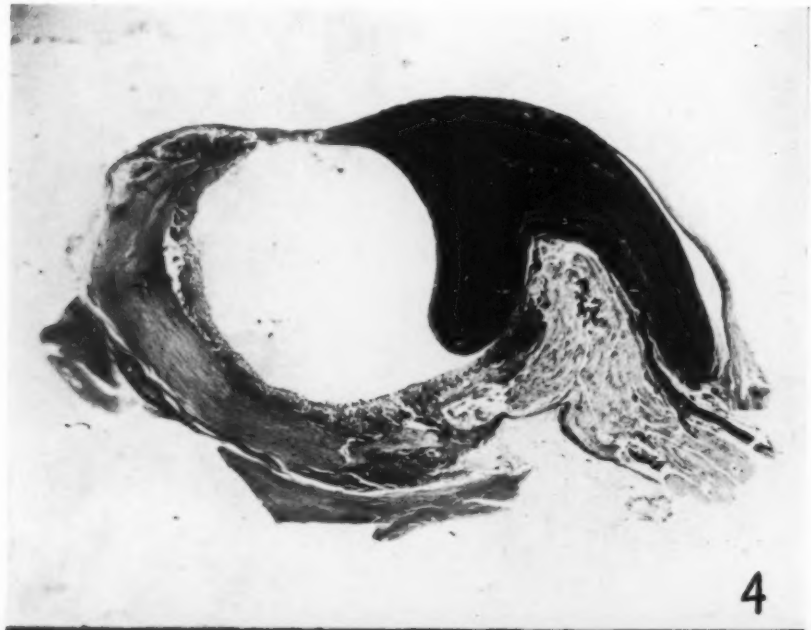
EXPLANATION OF FIGURES

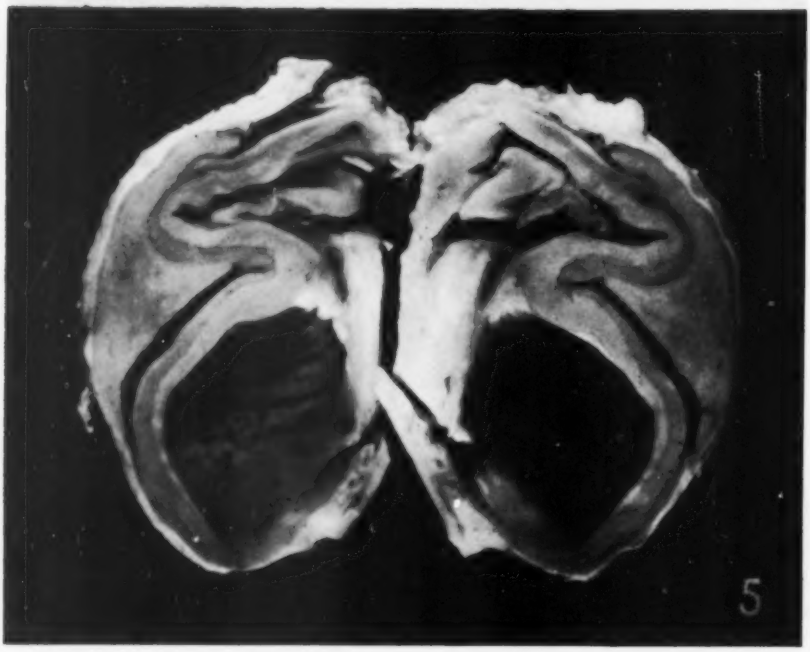
1 Section of anterior lobe of the pituitary from cow 234. × 5.8. Note position of cyst and large (light stained) area of pressure atrophy.

2 Near edge of pituitary from region A of figure 1. × 152. Note connective tissue replacement and atrophy of glandular cells.

3 Region B of figure 1. × 284. Note dense connective tissue stroma and nests of glandular cells, some of which are degenerating.







4 and 5 Sagittal section of pituitary from calves 166-1 and 173-1, respectively. Note location of large cyst in posterior lobe with compression of anterior lobe.

A STUDY OF THE ASCORBIC ACID REQUIREMENTS OF CHILDREN OF EARLY SCHOOL AGE [1]

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THREE FIGURES

(Received for publication February 18, 1942)

The amount of ascorbic acid required for normal nutrition is not, as yet, well established. More detailed studies are needed at all age levels. Standards for the infant have been estimated, for the most part, either by determining the amount of vitamin C it takes to prevent the development of infantile scurvy, or by comparing the blood levels of breast-fed and artificially-fed infants with the relative amounts of ascorbic acid provided by the milk. While well-controlled, quantitative studies have been made on preschool children and adults, investigations on the school-age child have been principally of the survey type. In such studies the vitamin C content of the diet is only estimated, usually by dietary records or by inventory methods. This study was undertaken with the view of contributing quantitative data on the vitamin C requirements of children of early school age.

EXPERIMENTAL

Selection of subjects. The subjects of this study were five children, two girls and three boys, between the ages of 7 and 12 years, living in a home for crippled and convalescent children. This institution was chosen because living conditions were such that it was possible to control all phases of the study. Of the children selected three had been under treatment for malnutrition, one for clubfoot, and one for rheumatic heart.

[1] This research was aided by the Talcott fellowship granted by Rockford College and the Omicron Nu fellowship awarded by the American Home Economics Association to the senior author.

Presented at annual meeting of American Institute of Nutrition, April 1, 1942.

In all cases treatment had been successful, and the children were judged by the physician to be in a normal state of health. They were being retained in the institution until home conditions were favorable for their dismissal. The daily routine and activity of these children were comparable to that in any home. The children attended school regularly and played outside at recess and after school.

The physical status of the subjects is summarized in table 1. It is noted that all were of approximately average weight, or above, and that all gained substantial amounts during the

TABLE 1

The physical status of subjects.

SUBJECT	AGE		SEX	CONDITION FROM WHICH RECOVERED	BODY WEIGHT	HEIGHT	PER CENT OVER- OR UNDER-WEIGHT [1]	GAIN IN WEIGHT DURING STUDY
	yrs.	*mos.*			*kg.*	*cm.*		*kg.*
M. G.	12	6	F.	Malnutrition	33.7	142.5	— 6	1.5
P. T.	11	10	M.	Rheumatic heart	43.6	147.5	+ 13	1.8
D. S.	10	3	F.	Malnutrition	31.0	138.0	— 3	2.5
R. M.	9	6	M.	Malnutrition	39.1	141.8	+ 12	2.3
L. M.	7	9	M.	Clubfoot	27.3	126.2	+ 4	2.2

[1] As judged by the Baldwin-Wood tables.

period of the study. The one who had had rheumatic heart gained 1.8 kg. in the 3 months. Weekly medical checks showed his heart condition to be completely controlled.

General plan of the study. The original plan for the study was to follow the saturation method used by Belser, Hauck and Storvick ('39) and to include, in addition, the determination of blood ascorbic acid. Since the initial blood levels of the children to be used in the study were found to be low (0.45 to 0.59 mg. per 100 cc.) it seemed wise to follow the changes in the blood levels as the intake was gradually increased, rather than to saturate the subjects at the beginning. The vitamin C content of the institutional diet was determined, therefore, and this amount used as the starting point for the investigation. To a basal diet providing 15 mg. of vitamin C, supplements of crystalline ascorbic acid [2] were added daily.

[2] We wish to express our appreciation to Hoffman-La Roche, Inc., Nutley, N. J., for furnishing the crystalline ascorbic acid.

The investigation included eight experimental periods of 7 days each. During these periods the supplements of ascorbic acid ranged from 40 to 130 mg. Starting with 40 mg. the daily supplement was increased 20 mg. each week until the blood levels remained practically constant with the increased intake. Thereafter the supplements were gradually decreased until the 50 mg. level was reached for four subjects, and 90 mg. for the fifth.

The amounts of reduced ascorbic acid in the urine and blood plasma were determined on each level of intake. At the end of each period a test dose of 300 mg. vitamin C was given. The amount excreted in the urine during the 24-hour period following this test dose was used as one criterion for judging the state of saturation of each subject. Blood levels, urinary "resting level" and "retention values" are other criteria employed in the final analyses.

Dietary control. Chemical analysis of the institutional diet showed that the vitamin C was provided, for the most part, by a small glass of fruit juice, one serving of fresh fruit, and a quart of raw milk. The rest of the food furnished little ascorbic acid, due probably to the methods of preparation employed. Lettuce and cabbage, for example, were shredded 5 or 6 hours before serving, and most vegetables were overcooked. Potatoes stood in water both before and after cooking. They were then well aerated in an electric mixer and kept on the steam table for a considerable time. As a result all the vitamin C was destroyed.

The diet, on the whole, met the usual standards for adequacy with the possible exception of thiamine and vitamin C. The low blood levels of ascorbic acid indicated a shortage of this vitamin, even though the diet provided an average of 50 mg. daily.

Few changes were necessary, then, in setting up the experimental diet. Fruit juices and raw fruits and vegetables were eliminated and replaced by canned fruits, juices, and vegetables of low vitamin C content. The basic diet which was planned to furnish 15 mg. ascorbic acid, was made up from the foods listed in table 2. The weighed foods were always given in the same amount, with the exception of the milk

which varied from 750 to 1000 gm., depending upon the vitamin C content of the rest of the diet.

Collection of urine. For quantitative work Todhunter and Fatzer ('40) and Richardson and Mayfield ('40) have pointed out the necessity of using 24-hour urinary excretions. During this investigation the daily urinary output was collected and analyzed for 3 or 4 days on each level of intake. In addition, analyses were made on the 24-hour collection following each test dose.

To preserve the ascorbic acid in the urine 5 N sulphuric acid and 8-hydroxyquinoline were used as suggested by Sendroy and Miller ('39). By using brown bottles for the urine, acidifying the samples immediately, and keeping them in the refrigerator, losses were kept within 2%.

TABLE 2

Foods used in the basal diet with amount of ascorbic acid contributed by each food.

FOOD	AMOUNT	ASCORBIC ACID	FOOD	AMOUNT	ASCORBIC ACID
Foods weighed	gm.	mg.		gm.	mg.
Fruit juices			Vegetables, canned		
Peach	120	0.7	Carrots	60	0.3
Pear	120	0.4	Beets	70	3.0
Plum	120	0.3	Beans, green	60	1.8
Apple	120	1.2	Beans, Lima	60	0.4
Dried fruit (stewed)			Potatoes, mashed	110	..
Apricots	80	2.9	Milk, raw	750–1000	7.5–12.0
Peaches	80	2.3	Cocoa	200	0.5
Pears	80	0.3	Cod liver oil	1 tsp.	..
Apples	80	0.2			
Raisins	80	0.2	*Foods served ad lib.*		
Prunes	80	0.3	Meats	daily	
Canned fruit			Beef		
Pear, Bartlett	80	0.8	Veal		
Juice	20	0.3	Lamb		
Cherries			Bacon		
(Royal Ann)	80	2.2	Chicken		
Juice	30	1.8	Eggs	4–6 times per week	
Plums (Lombard)	60	0.6			
Juice	30	0.4	Cereals	daily	
Grapes (white)	60	1.2	Bread	daily	
Juice	30	0.8			
Apple Sauce	100	0.7			

Chemical methods. Because of the impossibility of taking the photoelectric colorimeter to the institution it was necessary to use the titration method for determining ascorbic acid. Preliminary tests showed, however, that by the addition of a buffer to the acidified urine the rapid fading of the end-point was prevented and the results checked within 2% of the values obtained with the photoelectric colorimeter. The 2,6-dichlorophenolindophenol used as an indicator was standardized daily by the procedure of Menaker and Guerrant ('38). For food and urine analyses the methods of Bessey and King ('33) were used. The micro-technique with the Farmer and Abt apparatus ('36) was employed for all blood determinations. Fasting blood plasma separated from capillary blood was used, special care being taken to prevent any hemolysis.

All solutions were prepared with water redistilled in glass. A fluorescent daylight lamp was used as a source of light for titrating.

RESULTS AND DISCUSSION

As a basis for interpreting the results from these blood and excretion studies in terms of the requirements, three criteria have been used. These are based upon (1) the "retention" or "utilization" of ascorbic acid, (2) the response to a 300-mg. test dose, and (3) the concentration of ascorbic acid in the blood. Blood levels above 0.7 mg. per 100 cc., the level recommended as normal by Goldsmith and Ellinger ('39) and Wortis et al. ('38), have been used in this study to indicate a satisfactory state of nutrition in respect to vitamin C.

In addition to these three indices for estimating the ascorbic acid requirements, other methods are included in the discussion. These will provide a means of comparing the results with other reports found in the literature. Urinary "resting level," "saturation" as interpreted by Belser, Hauck and Storvick ('39) and blood levels higher than 0.7 mg. per 100 cc. have been included.

"Retention values." One method of interpreting the results from urinary excretion studies in terms of the requirements is based upon the "retention value," that is, the difference between the intake of ascorbic acid and that excreted in the urine. With preschool children, Hathaway and Meyer ('41)

demonstrated the constancy with which the body utilized ascorbic acid. They found the mean "retention" for four children to be 23 mg. on intakes ranging from 31 to 50 mg. This represented 74% of the intake at the 31 mg. level, the minimum intake on which maximum "retention" was obtained.

The tendency for the body to "retain" a nearly constant amount of ascorbic acid was observed with the subjects on this study. As shown in table 3 there is little variation in the

TABLE 3

Average daily urinary excretion and average "retention" of ascorbic acid on various levels of intake.

PERIOD	1	2	3	4	5	6	7	8	6	7	8	AVERAGE AND S. D.
Intake—mg.	55	75	95	115	135	105	85	65	145	125	105	
"Excretion" mg.												
M. G.	19	32	51	70	80	62	40	37				
P. T.	11	26	37	69	86	60	39	25				
D. S.	19	32	55	80	95	65	47	34				
R. M.	12	29	49	75	95	62	48	25				
L. M.	21	44	54	67	100				106	88	79	
Average	16	33	49	72	91	62	42	30				
"Retention"[1] mg.												
M. G.	36	43	44	45	55	43	45	28				42 ± 7
P. T.	44	49	58	46	49	45	46	40				47 ± 5
D. S.	36	43	40	35	40	40	38	31				38 ± 3
R. M.	43	46	46	40	40	43	37	40				42 ± 3
L. M.	34	31	41	38	35				39	37	26	35 ± 5
Average	39	42	46	43	43	43	43	35				

[1] Value does not include the two 24-hour specimen following the test dose

amounts "retained" by each individual on the different levels of intake tested. The values appear to be related to the weight of the children rather than to the age. They vary from 35 ± 5 mg. for L. M., the smallest subject, to 47 ± 5 mg. for P. T., the largest.

This relationship of intake to excretion and "retention" is further illustrated in figure 2. The daily urinary excretion of ascorbic acid varies in direct proportion to the intake. It is apparent, however, that the amount "retained" varies but

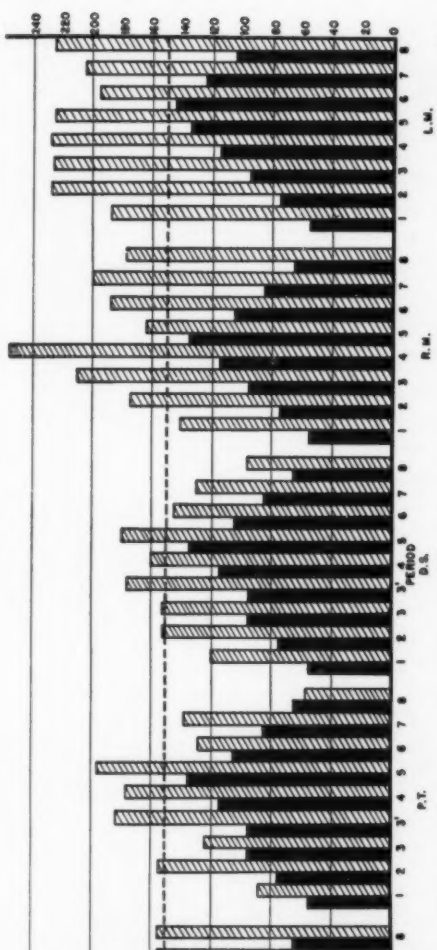

Fig. 1 24-hour excretion of ascorbic acid in response to a 300-mg. test dose following various levels of ascorbic acid intake. The black bars represent the previous intake, and the striped bars, the excretion after the test dose. 50% of the test dose is indicated by the broken line at the 150 mg. level.

Fig. 2 Average daily excretion and "retention" of ascorbic acid on various levels of ascorbic acid intake. The black bars represent the daily intake; the striped bars, the excretion; and the white bars, the "retention."

little. The lowest intake on which each subject "retained" an amount of ascorbic acid equivalent to the average "retention value" ranges from 55 mg. for two of the children to 75 mg. for the other three. The "retention" at these levels of intake represents 57 to 77% of the ascorbic acid ingested.

"Resting level." The "resting level" or amount excreted daily in the urine has been used as another index for measuring the state of vitamin C nutrition. Van Eekelen and associates ('37) considered a "resting level" of 40 mg. indicative of tissue saturation. The average daily excretions for the five subjects on this study are reported in table 3. The ascorbic acid in the urine varied directly in proportion to the intake. The average excretion increased from 16 mg. on an intake of 55 mg. of vitamin C to 91 mg. when 135 mg. was ingested. An output of 40 mg. resulted from an intake of 75 mg. for one subject and 85 mg. for the other four.

Response to a test dose. A more widely accepted measure of vitamin C nutrition is the so-called "saturation test." By this method a large quantity of ascorbic acid is administered — orally, subcutaneously, or intravenously — and the amount excreted in the urine determined. A large percentage return of the test dose within 24 hours has been interpreted as indicating saturation. The exact percentage used by different workers, however, varies in respect to the size of the test dose given, the method of administration, and the length of the collection period. For this study a 300-mg. test dose of ascorbic acid was given orally, and a 50% return in the urine during the subsequent 24-hour period was used as the criterion for judging saturation.

The relationship between the intake and the response to the test dose is illustrated in figure 1. The youngest subject, L. M., returned more than 50% of the test dose on the lowest level of intake, that is, 55 mg. On all other levels he remained saturated. Sixty-five milligrams were required by M. G. and R. M. to produce the same response. They, too, remained saturated on intakes above 65 mg. The results for P. T. and D. S., however, were variable. In each case 75 mg. was the

lowest intake upon which 50% of the test dose was excreted. It will be noted that P. T. excreted less of the test dose on the 95 mg. level than on the 75 mg. intake. Upon repeating the test, the percentage return was greater. Similar variations have been observed in studies on adults (Belser, Hauck and Storvick, '39; Todhunter and Robbins, '40; and Fincke and Landquist, '42). The figures from the present study are in agreement with the findings of Koch ('41). With children of early school age she found that her subjects required 52 to 72 mg. of ascorbic acid before they excreted 50% of a 300 mg. test dose.

In figure 1 it is shown, also, that P. T. and D. S. always excreted less of the test dose than the others. The procedure followed by Belser, Hauck and Storvick ('39) rules out to some extent these differences due to individual variations. In general, they completely saturate the subject and then determine his individual response to the test dose. The value thus obtained is used to indicate his saturation response. It is questionable whether the data from the present study may be interpreted on the basis of this technique inasmuch as the subjects were not saturated at the beginning. We may assume, however, that they were all saturated at the three highest levels of intake, for the ascorbic acid level of the blood remained constant. With this interpretation, where each child sets his own standard for response to the test dose, three of the subjects required the same amount of vitamin C for saturation as was needed when the criterion consisted of 50% return of the test dose; that is, P. T., D. S., and R. M. required an intake of 75, 75, and 65 mg., respectively, to produce saturation. For the other two children the results were higher; 95 mg. was needed by M. G., and 75 mg. by L. M. The latter subject was not tested on an intake of 65 mg.

Blood levels. The amount of ascorbic acid found in fasting blood is believed by some to be the most accurate measure of the state of vitamin C nutrition. Here again there is a wide disagreement as to the level which represents tissue saturation. Faulkner and Taylor ('38) and Ralli et al. ('39) agree

that the renal threshold for ascorbic acid is about 1.3 to 1.4 mg. per 100 cc. It is at this blood level that the tissues are saturated; levels below this indicate varying degrees of unsaturation. On the other hand, there seems to be little agreement as to how low the blood levels may go without serious nutritional effects. Goldsmith and Ellinger ('39) and Wortis, Liebmann and Wortis ('38) believe 0.7 mg. per 100 cc. to be normal. The committee on vitamins of the American Academy of Pediatrics (Butler et al., '40) suggests that a

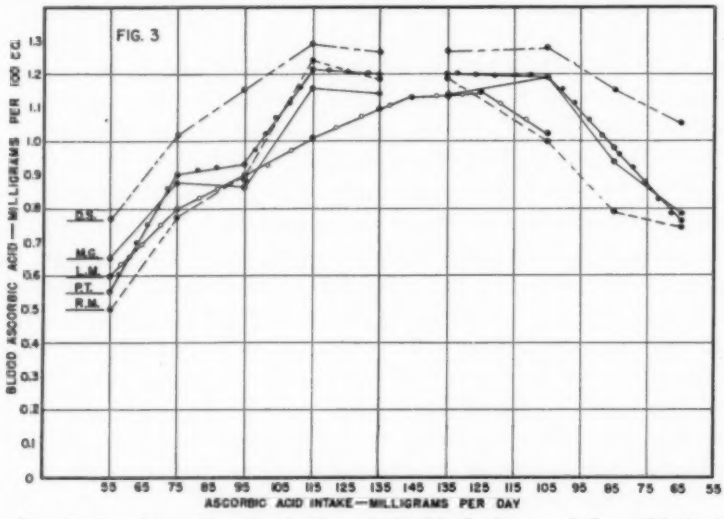

Fig. 3 Ascorbic acid concentration of the blood plasma of five subjects on varying levels of ascorbic acid intake.

serum or plasma concentration of 0.6 mg. or more per 100 cc. indicates a satisfactory state of vitamin C nutrition. Kajdi et al. ('39) believe that a blood level below 0.6 mg. per 100 cc. shows a serious depletion of body reserves. Whether these variations are due to differences in technique, or whether they are definite individual variations is difficult to determine.

At the beginning of the present study the blood levels of the children were between 0.5 and 0.6 mg. per 100 cc. The response of the blood plasma to graded amounts of vitamin C is illustrated in figure 3. The plasma values increased in

direct proportion to the intake until a plateau was reached at levels of 1.15 to 1.29 mg. per 100 cc. The lowest intakes which produced the maximum blood levels — the threshold value — were 105 mg. for three of the subjects, 115 mg. for the fourth, and 125 mg. for the fifth. As the intake was decreased below these amounts the plasma ascorbic acid likewise dropped.

To maintain blood values of approximately 0.9 mg. per 100 cc. the intake ranged from 65 to 95 mg. The two who required the highest intake to maintain this level, R. M. and L. M., were the ones who excreted the largest amount in response to the test dose. Also, their blood ascorbic acid rose more slowly than did that of the others.

On the lowest level of intake, 55 mg., the blood levels varied from 0.49 to 0.76 mg. per 100 cc. These individual variations in blood values were noted on all levels of intake. With adult subjects, Storvick and Hauck ('42) report variations even greater than these. If a blood value below 0.6 mg. per 100 cc. indicates a depletion of body reserves, then it would appear that the two largest subjects, P.T. and R.M., were depleted on the 55 mg. allowance. Only one subject, D. S., reached the plasma level of 0.7 mg. per 100 cc. on this intake. At least 65 mg. were required by all the others to bring the blood to 0.7 mg. per 100 cc., the concentration used in this study to indicate a satisfactory state of nutrition in respect to ascorbic acid. These results are in close agreement with those of Shelby ('41). In a study of thirty children, 6 to 12 years of age, she found that from 52 to 72 mg. were needed to maintain blood levels above 0.7 mg. per 100 cc. On the other hand, Bessey and White ('42) report that about 80% of their children had blood levels above 0.7 mg. per 100 cc. on intakes of 45 mg. This amount of ascorbic acid was estimated, however, on the consumption of citrus fruit and tomatoes only. Their results could better be compared then with the supplement alone used in the present study, and on this basis the results agree very well.

Of special interest are the blood levels corresponding to the "saturation" level. When a 50% excretion of the test dose

in 24 hours was used as the criterion for judging saturation, the blood ascorbic acid of the five subjects ranged from 0.60 to 1.05 mg. per 100 cc. These values are all below the renal threshold. In fact, some are below the levels suggested by some workers as being necessary for good nutrition.

COMPARISON OF REQUIREMENTS ON THE BASIS OF DIFFERENT METHODS OF ANALYSIS

The requirements as obtained by the several methods used in the interpretation of the data are summarized in table 4.

TABLE 4

Ascorbic acid requirements of five children as based upon various criteria for judging adequacy.

CRITERIA USED FOR JUDGING ADEQUACY	SUBJECT					
	M. G.	P. T.	D. S.	R. M.	L. M.	Average
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Intake to maintain average "retention" value [1]	75	75	75	55	55	67
Intake to maintain "resting level" of 40 mg. urinary excretion	85	85	85	85	75	83
Intake to insure "saturation"						
a. As indicated by 50% excretion of a 300-mg. test dose [1]	65	75	75	65	55	69
b. As indicated by method of Belser, Hauck, Storvick	95	75	75	65	75	77
Intake to maintain blood levels						
a. Of 0.7 mg. or above [1]	65	65	55	65	65	63
b. Of 0.9 mg. or above	75	75	65	95	95	81
c. Of maximum value	105	105	105	115	125	109

[1] The requirements for our subjects have been based upon these three indices.

Individually and collectively there is a wide range in the results. Including all methods of interpretation used, 65 mg. was the least value obtained for the requirements of the two oldest children, M. G. and P. T. Their blood levels were maintained above 0.7 mg. per 100 cc. at this level, and M. G. excreted 50% of the test dose in 24 hours. On all other bases, however, their requirements were higher. With the three

youngest children, 55 mg. was the least required. This was the lowest level providing maximum "retention" for two of the subjects. One of these, L. M., also excreted 50% of the test dose on this amount. Subject D. S. was the only child, however, who maintained a blood level above 0.7 mg. per 100 cc. when receiving 55 mg.

On the basis of the different criteria used it appears that an intake of 65 mg. for the two youngest children, and 75 mg. for the three oldest, would be sufficient to (1) maintain a blood level above 0.7 mg. per 100 cc., (2) provide for the maintenance of the average "retention" value and (3) insure saturation on the basis of a 50% excretion of a 300-mg. test dose. This represents a range of 1.7 to 2.4 mg. per kilogram of body weight. With other means of interpretation the requirements would be higher. Seventy-five to 85 mg., for instance, would be necessary to allow for a "resting level" of 40 mg. Amounts up to 95 mg. are needed to maintain blood levels of 0.9 mg. per 100 cc. In order to reach the threshold values these children required from 105 to 125 mg. of ascorbic acid.

SUMMARY

The vitamin C requirements of five children between the ages of 7 and 12 years were studied. Blood values and urinary excretion of ascorbic acid were determined on levels of intake ranging from 55 to 145 mg. Following each level of intake a 300-mg. test dose was given to determine the state of tissue saturation. The amount "retained" by the body, the blood concentration, and the response to the test dose were used as criteria for judging the nutrition of each subject in respect to vitamin C.

Sixty-five milligrams of ascorbic acid were required by the two youngest children, and 75 mg. by the three oldest, to promote saturation on the basis of a 50% excretion of the test dose in 24 hours. These amounts were sufficient, also, to maintain blood levels above 0.7 mg. per 100 cc., and to allow for average "retention." With other methods of interpretation the requirements varied from these amounts up to 125 mg.

ACKNOWLEDGMENT

To the children who served as subjects throughout this investigation we express our deepest appreciation. We wish to thank also, the entire staff of the Country Home for Convalescent Children of the University of Chicago for their fine cooperation.

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CHEMICAL COMPOSITION OF TWENTY-TWO COMMON FOODS AND COMPARISON OF ANALYTICAL WITH CALCULATED VALUES OF DIETS *

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(Received for publication February 19, 1942)

One of the greatest needs today when the science of nutrition is being put to a practical test is specific information upon the chemical composition of normal diets as they are eaten. In response to this need, the present paper has been designed to provide data which permit a comparison of the determined summation value of the individual fresh foods which make up the total daily diets with the analyzed mixed diets subsequent to culinary treatment and service (i.e., as eaten), and with the values calculated from standard food tables.

In a survey of the literature on the mineral content of common foods, Word and Wakeham ('38) have pointed out that, while the calcium, phosphorus and sulfur contents of some foods vary by only 25% or less, those of others may vary as much as 200%, when the analyses of different authors are compared. This statement need not imply that the validity of the analyses is questionable, but rather that there are differences in composition, even among specimens of the same variety of foods. In fruits (McCance and Lawrence, '29; McCance, Widdowson and Shackleton, '36), vegetables (Bishop, '34; Coleman and Ruprecht, '35; Davidson and LeClerc, '36), and cereal grains (Greaves and Hirst, '29; Schumpf-Pierron, '32), these discrepancies have been at-

* Some of the data in this paper were presented before the Division of Biological Chemistry of the American Chemical Society at the Ninety-Sixth National Meeting, Milwaukee, Wisconsin, September 5–9, 1938.

tributed to variety, type of soil and cultivation employed, amount of water available, and climate. Attempts to correlate variations in foods with several factors have been made but information is still fragmentary. Several investigators (Bassett and Van Alstine, '35; MacKay and Butler, '35; Hawks, Dye and Bray, '37; Gutman and Low, '39; Hummel, Shepherd and Macy, '40), describing the technique of conducting metabolic experiments, have emphasized the fact that because of the variability in the composition of foods, the diet given must be analyzed under the precise conditions of the experiment, if its mineral, or even its protein, contents are to be used with any assurance of accuracy.

This report presents the inconstancy in composition shown by chemical analyses of representative samples of twenty-two individual foods which were used in making up mixed dietaries for normal children: (1) a comparison of the determined chemical constituency with values calculated from standard tables of food values (Sherman, '41); and (2) a comparison of the records of intakes of carbon, nitrogen, fat, energy, cellulose, hemicellulose, lignin, calcium, magnesium, sodium, potassium, phosphorus, chlorine and sulfur, as calculated by summation of the values from chemical analyses of the individual foods, and as determined directly on composite food mixtures representing the total daily dietary.

EXPERIMENTAL

Sources of foods. Canned and packaged goods of the same brand (cornflakes, graham crackers, tomato juice, peanut butter and orange juice) were purchased in bulk. Perishable foods, such as lettuce, carrots, cabbage, and lean beef (ground shoulder), were purchased daily at a local market. Fresh white and whole wheat breads were purchased daily from one bakery. Wealthy (Michigan) apples and Michigan potatoes were used exclusively and procured from a single source. The milk supply was controlled by a creamery company [1].

[1] Mr. C. G. Reichle, plant manager of Borden Farm Products of Michigan, skillfully controlled the handling of all the milk used in the analyses.

Preparation of individual foods for analysis. Samples of each of the individual foods were dried in a manner designed to cause the least possible alteration of their constituents. Solid foods were dried in vacuo at 40° C.; liquid foods were dried from the frozen state in the cryochem apparatus (Flosdorf and Mudd, '38) under vacuum at pressures of 150–200 microns. After the solid foods had reached constant weight, the samples were pulverized in a ball mill and stored in a desiccator until analyzed.

Preparation of food mixtures for analysis. The preparation of the separate foods, including sampling and preparation for analyses, has been described elsewhere (Macy, '42; Teague, Galbraith, Hummel, Williams, and Macy, '42). To obtain an accurate sampling of the foodstuffs, within the range of variation in composition usually occurring in practice and representative of the average American dietary, foods obtained at different seasons during several years were analyzed. All but five of the foods used (sugar, salt, milk, potatoes, butter) were incorporated in mixed composite samples of the diets, although milk was included in one composite. The salt used was chemically pure NaCl and the sweet butter given was thoroughly washed with distilled water. Sugar, salt and butter were not analyzed but their values were added to those for the composite. In most instances milk and potato were analyzed separately and the values added to those for the food mixture.

Food mixtures which would represent actual daily diets were prepared (Bassett and Van Alstine, '35). Each day samples of every food in the diet were taken and placed in separate, covered glass jars; each sample approximated two-fifths of the total daily intake of that food. At the end of 5 successive days each jar contained both a mixture of one food equal to approximately twice the daily intake in quantity, and a sample from each day's intake. From the food jars two identical composites were prepared, each representing a complete dietary for 1 day. One of the duplicate composite samples was transferred to an evaporating dish, and dried to

constant weight at 60° C. in the presence of alcohol. The dry material was ground in a ball mill, which insured thorough mixing, and used for determinations of the calcium, magnesium, potassium, sodium, phosphorus and chlorine contents. The second sample of the complete dietary was ground in a food chopper, and one aliquot of the homogeneous mixture removed for the determination of nitrogen; this aliquot was digested with sulfuric acid and made to volume. Another aliquot, about one-tenth of the total, was dried to constant weight in vacuo at 40° C., after which the material was ground in the ball mill and used for the determination of sulfur, energy, fat, carbon, lignin, cellulose and hemicellulose.

Methods of analyses. The methods employed in the analyses were used as published by Macy ('42); nitrogen was determined by the boric acid modification of the Kjeldahl method (Scales and Harrison, '20); calcium was precipitated as the oxalate from acetic acid solution (Scott, '39); magnesium was precipitated from the filtrate remaining from the calcium determination, under the conditions suggested by Willard and Furman ('35); sodium was precipitated as sodium uranium zinc acetate, using Butler and Tuthill's ('31) modification of the Barber and Kolthoff method; the Kramer and Tisdall ('21) method for the determination of potassium was followed as described by Peters and Van Slyke ('32); phosphorus was determined gravimetrically by MacKay and Butler's modification of Mathison's method (Peters and Van Slyke, '32); chlorine was determined gravimetrically as silver chloride (Garelli, '32); and, sulfur was precipitated as barium sulfate, after the food samples had been oxidized in the Parr Adiabatic Oxygen Bomb [2] and heat of combustion determined. Fat was extracted from the vacuum dried material in a Soxhlet apparatus. Carbon was determined by the macro wet-combustion process (Scott, '39). Lignin, cellulose and hemicellulose were determined by the method of Williams and Olmsted ('35).

[2] Parr Oxygen Bomb Calorimeters and Oxygen Bomb Sulfur Apparatus, Manual No. 117, Parr Instrument Co., Moline, Illinois.

Iron was determined by the o-phenanthroline method devised by Hummel and Willard ('38).

RESULTS AND DISCUSSION

The mean values from the determinations for the energy, fat, carbon, nitrogen, calcium, magnesium, potassuim, phosphorus, sodium, chlorine, and sulfur contents per 100 gm. of edible portion of each of the twenty-two foods used in the diets are summarized in table 1. The widest variations from the standard tables were found in the determinations of sodium and chlorine. Since NaCl is a component of extracellular fluid in plant and animal tissues, the sodium and chlorine contents might be expected to be influenced to a greater extent by the composition of the soil and water in which the plant materials were grown, and by the degree of dehydration. Potassium, which is an intra-cellular constituent, differed less from previously reported values than any other mineral constituent determined. Greater consistency among intra-cellular constituents is further supported by the phosphorus and sulfur [3] determinations, which also are in good agreement with values previously reported.

Since standard tables are usually compiled from the averages of large numbers of determinations from widely different sources, it is of interest to determine whether the mean values of the two foods for which the largest number of samples were analyzed (banana, 14; milk, 25) approach more closely the values reported in the standard tables. Analyses of more samples would be expected to include a wider range of variability and yield mean results in closer agreement with the standard tables; conversely, a single sample might be an extreme variation from the mean of many determinations and would then have little significance, except with reference to the experiment in which the food was used.

[3] Since the amounts of sulfur were exceedingly small, greater accuracy was attained by determining and applying to the data a correction factor for the solubility of barium sulfate in water (which was used as a wash solution).

TABLE 1

Variation in analysis content of individual foods[1] *(values in units per 100 gm. edible portion).*

	ENERGY	FAT	CARBON	NITROGEN	CALCIUM	MAGNESIUM	POTASSIUM	SODIUM	PHOSPHORUS	CHLORINE	SULFUR
	Cal.	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Apples (not peeled)	55	0.88	5.4	0.04	6	8	125	2	13	2	4
Banana (peeled)	86	0.24	8.7	0.13[2]	5[2]	33[2]	387[2]	5[2]	28[2]	110[2]	8[2]
Beef	184	8.06	22.0	3.14	10	13	290	70	148	63	162
Bread (white)	290	1.36	27.6	1.56	60	28	107	601	114	843	116
Bread (whole wheat)	283	1.51	28.4	1.68	64	67	203	601	217	857	126
Cabbage	24	0.12	2.4	0.21	44	12	160	24	16	...	64
Carrot	43	0.16	4.1	0.10	26	18	320	58	30	144	17
Cheese (Velveta)	350	19.90	30.0	3.05	565	15	255	1440	875	1122	178
Corn flakes	384	0.25	37.8	1.14	7	37	98	1120	42	1633	115
Egg	146	7.32	12.8	1.70	42	2	120	128	165	124	155
Graham Cracker	372	4.34	36.6[2]	1.12	46	62	384	670	157	516	116
Lettuce	24	...	2.0	0.14[3]	16	12	172	16	20	...	12
Milk (evaporated)	...	...	...	0.97	253	25	294	138	209	224	68
Milk (fluid)	70[2]	3.45[2]	6.2[2]	0.50[2]	118[2]	11[2]	148[2]	50[2]	90[2]	108[2]	30[2]
Orange Juice (fresh)	40	...	5.2	0.15	11	11	187	6	15	8	...
Orange Juice (canned)	...	...	49.1[2]	...	12	12	192	4	24	6	6
Peanut Butter	632	45.20	10.0[2]	4.64	28	178	694	384	341	616	225
Peas	96	0.44	7.7	1.10	22	32	296	98	114	187	41
Potatoes (peeled)	76[2]	0.10	...	0.35	8[2]	22[2]	394[2]	...	60[2]	34[2]	41[2]
Spinach (canned)	...	...	2.2	0.29	53	63	208	378	344	460	...
Tomato Juice (canned)	20	...	43.3	0.13	3	10	225	255	17	387	13
Honey	293	...	69.0	...	...	...	...	...	...	...	13
Gelatin	467	...	28.7	16.20	...	...	...	...	...	...	467

[1] Unmarked figures are average of five, (or fewer) triplicate analyses of samples.

[2] Average of six to fifteen triplicate analyses of samples.

[3] Average of fifteen to twenty-seven triplicate analyses of samples.

The food most constant in composition, in our experience, both with respect to variation within a given series, and by comparison of our results with those of others, is fluid cow's milk. Analyses agree with Sherman's standards within 3% for energy, calcium, phosphorus, potassium, sodium, and chlorine; magnesium and sulfur, which are present in extremely small quantities in milk, agree within —8 and —12%, respectively; nitrogen and fat agree within —11 and —12%, respectively. The samples of milk analyzed represented pooled samples from one herd of cows and the results of the analyses emphasize the value of careful control of the source of a material to be analyzed for mineral composition; furthermore, a pooled sample might be expected to approach the previously determined mean figures more closely than a sample from a single animal. The constancy of milk might be predicted from the fact that it is a body secretion; however, it is very susceptible to contamination, a factor which has been frequently mentioned as a possible reason for variability in food analyses. The analytical value for milk composition indicates that either contamination of the samples was of minor importance in introducing errors in the analyses or the contamination factor is about the same for all milks analyzed, including those reported herein and by others.

Banana, which is protected by a skin, making the fruit practically free from contamination, varied widely in composition in spite of the fact that the fruit for the analyses reported in this paper was selected for uniform quality and ripeness by an expert trained in handling this type of fruit [4]. The standard deviations of the analyses of banana demonstrate a wide range of variability for several constituents, in spite of their protective covering, and the average values differ widely from Sherman's figures. The determined values agreed with those of Sherman within 6% for phosphorus, magnesium and potassium and 20% for fat; —12% for energy and

[4] Mr. B. E. Reiff, resident manager, Fruit Dispatch Company, Detroit, assumed the responsibility of selecting and delivering the bananas so that they would be at comparable stages of ripeness when analyzed.

chlorine; —33% for nitrogen and sulfur; —38% for calcium; and —88% for sodium.

The fat contents of the foodstuffs analyzed varied rather widely from the standard figures. The few foods, other than butter and milk, which contained fat in appreciable amounts were concentrated types of foodstuffs which are difficult to sample accurately. In addition, they were not uniform as purchased, though every possible precaution was observed to control variations which might arise at the source of supply. For example, shoulder beef was recommended as the most uniformly lean meat to be obtained and all purchases were made from one butcher, but samples were found to vary as much as 1 to 15% in fat content. The energy values of the beef confirmed the difference in composition.

Table 2 illustrates the variability of lignin, cellulose and hemicellulose analyses of individual foods. Although the shortcomings of the methods for determining the complex carbohydrates are recognized, the values recorded do have merit in that our knowledge of these constituents in the human dietary is so fragmentary. There are no analogous figures in the literature with which to compare our values.

Table 3 permits comparison of the sum of the values for individual foods in the diets with the determination of the same constituent on the mixed dietary composite as eaten; in addition, the quantity of each dietary constituent is shown as calculated from standard tables. The data for the mixed composite are means of eleven triplicate analyses. The standard deviations (S. D.) have been calculated and recorded. The sum of the analyses of individual foods is within a range of 1 S. D. from the mean of the analyses of the composite, for fat, nitrogen, magnesium, sodium, potassium, chlorine, and energy; for calcium, phosphorus, and sulfur the sum of the individual analyses is more than 1 S. D. below the mean. The calcium intakes calculated from the standard tables are significantly higher than the determined values, while the sodium and chlorine intakes are decidedly lower.

TABLE 2

Complex carbohydrates of individual foods (milligrams per 100 gm. of edible portion).

	NO. SAMPLES	LIGNIN			CELLULOSE			HEMICELLULOSE		
		Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.
Apple[1] (not peeled) (Wealthy)	2	170	136	203	514	460	568	200	197	203
Banana (peeled)	3	416	344	462	153	60	234	125	105	154
Bread (white)	3	71	68	76	92	0	275	494	444	534
Bread (whole wheat)	3	451	290	592	627	470	740	1362	1060	1723
Cabbage[1]	3	87	60	104	531	504	552	385	360	428
Carrot[1] (scraped)	3	97	64	114	676	608	784	517	368	682
Cornflakes	3	184	117	223	599	453	757	366	327	430
Graham Cracker	3	299	178	433	460	264	567	741	383	1056
Lettuce	3	83	80	84	367	340	412	242	215	260
Peanut Butter	3	95	36	212	323	100	600	762	531	931
Peas	3	65	48	84	2049	1856	2340	707	548	944
Potatoes[1] (peeled)	9	31	7	72	246	0	417	110	33	273
Spinach (canned)	1	128	128	128	286	286	286	142	142	142
Tomato Juice (canned)	2	8	7	10	126	110	143	34	27	42
Shredded Wheat	1	797	797	797	1650	1650	1650	3473	3473	3473

[1] Michigan.

TABLE 3

Chemical composition of diet as determined by analysis of composites, analysis of the individual foods, and by calculation from standard tables.

	VALUES FROM ANALYSIS OF FOOD COMPOSITE		PRECISION [1] OF CHEMICAL METHODS	SUM OF VALUES FOR INDIVIDUAL FOODS		PER CENT DIFFERENCE COMPARISON	
	Mean	SD		Determined	Calculated [2]	I [3]	II [4]
			%				
Energy (Cal.)	1429 [5]	78	1.3	1448 [5]	1398 [6]	+4	+1
Fat (gm.)	35.52	2.72	1.6	34.91	40.29	−13	−2
Protein (N × 6.25) (gm.)	64.5	2.8	..	62.8	65.6	−4	−3
Nitrogen (gm.)	10.32	0.45	0.3	10.04	10.49	−4	−3
Calcium (gm.)	0.347	0.014	3.6	0.312	0.434	−28	−11
Magnesium (gm.)	0.228	0.014	3.8	0.224	0.255	−12	−2
Sodium (gm.)	1.933	0.036	1.0	1.962	0.789	+149	+1
Potassium (gm.)	2.283	0.063	1.1	2.299	2.374	−3	+1
Phosphorus (gm.)	0.981	0.041	2.4	0.923	1.109	−17	−6
Chlorine (gm.)	2.582	0.088	1.1	2.522	1.081	+133	−2
Sulfur (gm.)	0.771	0.054	2.6	0.698	0.706	−1	−10
Total positive minerals (meq.[7])	178.6	....	..	178.2	137.7	+29	−0.22
Total negative minerals (meq.[7]) .	177.9	....	..	168.3	139.0	+21	−6
Excess of positive minerals (meq.[7])	0.7	....	..	9.9	−1.3	....	....

[1] Determined by Analysis of Variance (Snedecor, '34).

[2] From Sherman ('41).

[3] Sum of individual foods as analyzed vs. sum of individual foods as calculated.

[4] Sum of individual foods as analyzed vs. analysis of composite.

[5] Heat of combustion.

[6] Fuel value (Sherman, '41).

[7] Milliequivalents.

In general, a fairly large constituent, such as potassium in plant materials, or calcium in milk and cheese, can be determined with relatively greater accuracy than a very small one, such as calcium, magnesium, phosphorus and sulfur in most foodstuffs. Analysis of composite diets decreases the chances of error from weighing and handling and conserves the time of the laboratory staff, resulting in a great reduction of expense, although physical or chemical characteristics of some foods indicate the advisability of separate analysis and addition of the individual values to the data for the composite. The wide variations that occur in the composition of individual foods emphasize the advantages of analyzing composite diets.

In composition, the composite samples were subject to less than the usual number of variations, although changing season necessitated the purchase of foods of different variety, grown under different conditions of soil and climate. Possibilities that must be considered are (a) minor differences in weights from one period to another; (b) varying degrees of dehydration due to storing the foods, both in market and refrigeration; and (c) inherent personal errors by the persons through whose hands each sample passed before it was ready for analysis. The precision of the chemical methods has been calculated and is recorded in table 3. In many series of analyses the accuracy of a micromethod was approached.

Comparison of composite diets. Table 5 gives summaries from eleven constituents of the six mixed dietaries itemized in table 4. The number of samples analyzed, the range of the determinations, and the mean, with its standard deviation and the standard error of the mean, are given. In a recent paper, Hawks et al. ('40) have indicated that the errors inherent in analyses of composite diet preparations are from sources other than manipulation of the foods. The standard error of the mean in the determination of nitrogen (table 5) is of the same order of magnitude as the errors found by these investigators when foods weighed on the same day were analyzed. This is particularly significant when it is remembered that each mean given in table 5 presents the average

of successive determinations of 5-day composite diets which were collected over several weeks at a time. The determinations of calorie content of the diets likewise have practically the same range as those of Hawks and associates ('40), thus supporting their conclusion that any errors found in analyses of composite diets are from a source other than sampling and manipulation.

The analytical results presented for six dietary mixtures indicate that the accuracy of sampling and chemical methods

TABLE 4

Distribution of foods in the mixed diet composites [1]

FOOD	AMOUNTS OF INDIVIDUAL FOODS IN COMPOSITE DIETS Grams					
	A	B	C	D	E	F
Apple	100	100	100	100	100	100
Banana	100	...	...	...	...	150
Beef, lean	60	100	100	100	100	100
Bread, white	60	20	10	70	60	50
Bread, whole wheat	30	30	30	30	30	50
Cabbage	50 [2]	25	25	25	25	25
Carrot	50 [2]	25	25	25	25	50
Celery	20	..	..	..	..	..
Cornflakes	..	30 [2]	10 [2]	30 [2]	10 [2]	30
Shredded wheat	30	30 [2]	10 [2]	30 [2]	10 [2]	..
Cheese, American	15	15	15	15	15	20
Egg, whole	50	50	50	50	50	100
Graham cracker	..	36	36	36	36	36
Gelatin	..	..	..	..	..	3
Honey	..	..	..	..	..	15
Lettuce	20	20	20	20	20	25
Milk	400	..	..	..	..	..
Orange juice	100	..	..	..	..	100
Peas	..	..	..	..	..	25
Peanut butter	..	16	16	16	16	16
Potato	70	40	40	70	70	..
Tomato juice	60	60	60	60	60	60

[1] Sugar, salt and butter were not included in the composites. The salt used was chemically pure NaCl; fresh, sweet butter was thoroughly washed with distilled water. In some of the composites, banana, orange juice, milk and potato were omitted, but these foods were analyzed separately and the values added to those for the composite.

[2] Cabbage and carrot were interchangeable in composite A. Cornflakes and shredded wheat were interchangeable in composites B, C, D, and E.

TABLE 5

Chemical composition of mixed composite[1] diets as eaten.

	ENERGY[2]	FAT	NITROGEN	CALCIUM	MAGNESIUM	SODIUM	POTASSIUM	PHOSPHORUS	CHLORINE	SULFUR	IRON
	Cal.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*		*mg.*
Composite A											
Samples[3]	10	...	20	20	20	20	20	20	20	20	...
Minimum	1097	...	8.03	0.903	.274	1.151	2.107	1.253	1.674	.667	...
Maximum	1405	...	9.08	1.052	.332	1.702	2.604	1.543	2.541	.981	...
Mean	1258	...	8.44	0.989	.310	1.368	2.324	1.348	2.060	.756	...
SD	90	...	0.12	0.037	.008	0.180	0.153	0.075	0.298	.082	...
SE_m	28	...	0.03	0.008	.002	0.040	0.034	0.017	0.067	0.18	...
Composite B											
Samples[3]	12	15	12	12	13	13	12	11	11	12	11
Minimum	857	23.4	7.02	.223	.152	0.788	1.124	.581	1.174	.435	6.38
Maximum	957	32.0	7.84	.254	.185	1.004	1.348	.635	1.358	.537	7.29
Mean	894	27.1	7.37	.240	.166	0.892	1.242	.610	1.272	.494	6.89
SD	22.5	2.6	0.28	.016	.010	0.061	0.078	.019	0.057	.029	0.28
SE_m	6.5	0.7	0.08	.005	.003	0.017	0.022	.006	0.017	.008	0.08
Composite C											
Samples[3]	12	12	12	11	12	12	11	11	11	11	11
Minimum	754	25.4	6.78	.209	.128	.694	1.133	.505	0.975	.428	5.92
Maximum	903	31.9	7.39	.266	.163	.854	1.348	.564	1.118	.530	6.69
Mean	805	27.3	7.06	.237	.147	.781	1.236	.537	1.053	.472	6.27
SD	30.2	1.9	0.34	.017	.012	.051	0.080	.012	0.042	.039	0.31
SE_m	8.7	0.6	0.10	.005	.003	.015	0.024	.004	0.013	.012	0.09
Composite D											
Samples[3]	17	20	15	16	17	17	17	17	16	15	15
Minimum	1014	25.5	7.88	.250	.170	1.051	1.267	.632	1.580	.517	6.82
Maximum	1112	34.0	8.50	.316	.207	1.267	1.571	.739	1.709	.597	8.91
Mean	1059	29.3	8.24	.284	.186	1.192	1.430	.689	1.653	.572	7.69
SD	37.7	2.7	0.06	.024	.013	0.054	.096	.039	0.039	.031	0.61
SE_m	9.2	0.6	0.02	.006	.003	0.013	.023	.009	0.010	.008	0.16
Composite E											
Samples[3]	5	5	6	6	6	5	6	5	5	5	5
Minimum	921	27.2	7.62	.264	.164	1.045	1.316	.567	1.465	.500	6.80
Maximum	1001	33.3	8.10	.281	.184	1.094	1.422	.682	1.706	.606	8.04
Mean	966	30.8	7.87	.270	.174	1.075	1.371	.635	1.546	.544	7.23
Composite F											
Samples[3]	11	11	11	11	11	11	11	11	11	11	...
Minimum	1341	31.9	9.62	.318	.219	1.860	2.192	0.921	2.327	.664	...
Maximum	1573	41.4	10.79	.375	.242	2.003	2.355	1.036	3.085	.845	...
Mean	1433	35.5	10.31	.347	.229	1.927	2.286	0.979	2.568	.771	...
SD	78.1	2.7	0.31	.024	.008	0.060	0.052	0.034	0.223	.051	...
SE_m	23.6	0.8	0.09	.007	.002	0.018	0.016	0.010	0.067	.015	...

[1] Amounts of the individual foods in each composite are given in table 4.

[2] Heat of combustion.

[3] Triplicate analyses were obtained for each sample.

were comparable. Composite A, however, had the highest accuracy with respect to calcium and phosphorus analyses. The fact that this mixture was the only one which contained milk, and thus possessed relatively greater quantities of these two elements, undoubtedly helps to explain the greater accuracy of these determinations. The number of samples analyzed increases the accuracy of all the mean values; since, as the number of samples analyzed is increased, the range of variation of the constituent foods is more nearly included, and a true mean figure approached. This is supported by the results obtained for composite A, which represents analyses of twenty separate diets, and D, which is based on seventeen analyses of composites. Composite D had the smallest standard error of the mean for nitrogen, chlorine, sulfur and fat.

SUMMARY

1. The analyses of twenty-two common foods for nitrogen, fat, energy, the positive (calcium, magnesium, sodium and potassium) and negative (phosphorus, chlorine and sulfur) minerals and iron indicate that individual samples of a given food vary from values reported in the standard tables.

2. Fruits and vegetables vary widely while milk is more constant in mineral content.

3. The variability of common foods does not seem to be as much a measure of contamination as a determination of real differences in composition.

4. Comparison of a series of analyses of eleven composite diets with the sums of the corresponding values for the individual foods in the diet, emphasizes the increased accuracy which may be obtained when a larger amount of a given constituent is contained in the material available for analysis. The composite diets showed a more constant composition than the components.

5. When the analyses of composite diets are compared with dietary figures calculated from the literature, there is good agreement in the content of magnesium, potassium, phosphorus, sulfur, calories and fat. Sodium, chlorine and calcium may be significantly different from calculated values.

6. If mineral, energy or fat content of a composite diet or an individual food is to be known with the highest degree of accuracy, it should be analyzed under the conditions of the experiment in which it is to be used.

7. The variations from time to time, as indicated by the standard error of the mean of each series, did not vary appreciably for a given constituent excepting in the case of sodium and chloride, even when the technique was refined in every possible manner. This indicates that the variations are inherent in the foods themselves rather than measures of errors in manipulation.

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THE UTILIZATION OF CAROTENE AND VITAMIN A IN THE RAT

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(Received for publication January 26, 1942)

Attention in recent years has been directed towards those factors influencing the availability of carotene and vitamin A. The method generally conceded to be the most suitable for such studies is based upon the determination of the vitamin in the livers of rats previously fed equivalent amounts of carotene or vitamin A from different sources; the conversion of carotene or the vitamin into liver stores of the latter when fed in excess of the daily requirement being made the measure of utilization.

The results of previous investigations have shown conclusively that the principal storage center for vitamin A in the rat is the liver (Sherman and Boynton, '25; Moore, '31; and Baumann, Riising and Steenbock, '34).

That a relationship between the availability of dietary vitamin A potency and the liver storage of vitamin A is valid, has been amply demonstrated by Davies and Moore ('34, '35), Greaves and Schmidt ('35), Lease, Lease, Steenbock and Baumann ('39), Gray, Hickman and Brown ('40), and Smith and Otis ('41).

It becomes increasingly important that further information be obtained on those factors that might affect the significance of results obtained from studies on the storage of vitamin A in the liver and the relative availability of carotene and vitamin A for this purpose under controlled experimental conditions. It was with these objects in view that the herein-reported experiments were carried out.

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EXPERIMENTAL

The storage of vitamin A in the rat livers was measured by means of a Bausch and Lomb medium quartz spectrograph by a method already described by Fraps and Kemmerer ('38).

In all the experiments male rats were used, and each replicate contained litter mates of approximately the same weight. Distilled water was given and, except where otherwise noted, diet 678-B (table 1) which is vitamin A-free. In all cases care

TABLE 1

Ingredients of diets used.

	678-B	7027-A
	%	%
Heated cornstarch [1]	59.0	
Heated casein [1]	22.0	
Irradiated yeast	10.0	0.53
Salt mixture no. 7009	4.0	
Sodium chloride	1.0	0.50
Wesson oil	4.0	3.9
Cornmeal (white)		49.5
Cottonseed meal		9.7
Powdered skim milk		29.1
Wheat bran		1.9
Non-irradiated yeast		4.3
Calcium citrate		0.5
Ferric chloride		0.05
Copper sulphate		0.02

[1] Cornstarch and casein heated at a temperature of 110° C., 24 hours.

was taken to equalize the food intake between litter mates. In order to eliminate food wastage the diet was moistened to a paste-like consistency prior to feeding.

In the first experiment a comparison was made of the vitamin A storage in the livers of rats that received equivalent amounts of vitamin A potency, as found in cod liver oil, carotene dissolved in cottonseed oil, and carotene as contained in alfalfa leaf meal. Four groups of rats containing eight replicates were used. The animals were placed on the experiment when 28 days old, the time they were weaned. From the time the young were born until weaning, the females received, ad

libitum, the regular stock diet for nursing females (diet 7027-A, table 1), which has a very low vitamin A potency.

The rats in group 1, the negative controls, received daily 0.1 ml. of cottonseed oil as a supplement. Rats in group 2 received daily 0.1 ml. of cottonseed oil containing 60 I. U. of vitamin A potency in the form of 36 μg. of carotene purified by precipitation with methyl alcohol (Fraps and Kemmerer, '39). The rats in group 3 received daily enough alfalfa leaf meal of high carotene content to give 60 I. U. of vitamin A potency as determined by the method of Fraps and Kemmerer ('39). The rats in group 4 received daily 60 I. U. of vitamin A from U. S. P. XI reference cod liver oil, dissolved in 0.1 ml. of cottonseed oil. All rats were on experiment for 14 days, then killed, their livers removed, and the vitamin A content determined spectrographically for each liver. In all the experiments, two or more litter mates were killed at the time of weaning, and the vitamin A content of their livers determined. All results are expressed in total micrograms of spectro vitamin A found in each liver.

In the second experiment, designed to show the effect of time of depletion on the vitamin A stored in the weaning rat's liver, the same general methods of selection and analysis were followed, except that two groups of eight replicates were used. The rats in group 1 were on experiment 2 weeks, and those in group 2 for 4 weeks. All rats received the basal diet (678-B), food intakes being equalized between litter mates; the same level of food intake was used for the surviving rat in the replicate during the last 2 weeks of this experiment.

In the first and second experiments it was observed that an increase occurred in the spectro vitamin A content of the livers even when the rat received a diet devoid of this vitamin. The third experiment was designed to show the causes of this increase. Three groups of rats consisting of eight replicates were placed on experiment when they were 21 days old. The feeding period was 7 days. The rats in group 1 received diet 678-B; the rats in group 2 received a similar diet but with the yeast replaced with an equal weight of cornstarch. Thia-

mine hydrochloride was fed daily to the rats in group 2 in sufficient amount to cover their daily thiamine requirements. The rats in group 3 were fed the stock diet (7027-A). The data obtained from all the experiments were analyzed statistically by the method of analysis of variance as given by Snedecor ('38).

RESULTS

The data obtained from the first experiment are presented in table 2. An increased retention of vitamin A is found in all diet groups over the values for the controls at the beginning. Those rats receiving vitamin A showed greatest retention of the vitamin, the animals receiving carotene in

TABLE 2

Effect of different sources of vitamin A potency on the spectro vitamin A content of rat livers.

	NO. OF RATS	SPECTRO VITAMIN A PER LIVER, IN MICROGRAMS		
		Range	Mean	Standard deviation of mean
Contents at beginning	25	2.5– 18.2	8.2	3.7
Contents at end	9	21 – 39	29.7	5.8
Carotene in cottonseed oil[1] (36 μg.)	9	42 – 88	67.0	15.4
Carotene in alfalfa (36 μg.)	9	29 – 63	42.7	12.0
Vitamin A in cottonseed oil[1] (60 I.U.)	9	70 –121	92.9	17.0

Difference between litters is significant; difference between diet groups is significant.

[1] Wesson.

cottonseed oil gave a lower retention, and those fed carotene in alfalfa leaf meal gave the lowest retention. The differences between diet groups and between replicates are statistically significant. Assuming the efficiency of vitamin A from cod liver oil to be 100% for liver storage purposes, carotene in cottonseed oil was found to be 59% as efficient, and carotene as found in alfalfa leaf meal was 21% as efficient at the potency level fed. Carotene as found in alfalfa leaf meal was 35% as efficient for vitamin A storage purposes as was carotene

dissolved in cottonseed oil. No account was taken of the possible retention of the carotene in the liver as such, since previous observations have shown that such retentions at levels similar to those fed are so slight as to be negligible.

It is observed that the negative controls also showed an increase in the vitamin A content of the liver over that found in the litter mates killed at the start of the experimental feeding; it is apparent that some substance must have been present in the vitamin A free diet (678-B) that is retained in the liver and gives a false vitamin A value upon spectrographic analysis. However, the relative retentions remain valid with the type of experimental design used for these studies.

TABLE 3

Effect of length of time of depletion on the spectro vitamin A content of rat livers.

	NO. OF RATS	SPECTRO VITAMIN A PER LIVER, IN MICROGRAMS		
		Range	Mean	Standard deviation of mean
At beginning	13	8.4–30.8	15.1	6.4
Depleted 2 weeks	7	19.4–27.5	24.3	3.2
Depleted 4 weeks	7	20.9–31.9	26.1	3.9

Difference between beginning and second week is significant; difference between second and third week is not significant.

The data presented in table 3 summarize the results obtained in the second experiment. A definite increase is observed in the liver stores of all rats maintained on the vitamin A free diet (678-B) for a period of 2 weeks or longer; this increase is significant. No significant increases occurred in the liver store of vitamin A in those rats continued on diet 678-B for an additional 2 weeks, as compared with their litter mates killed at the end of the first 2-week period. Whether the liver reached a saturation point for the substance giving the false vitamin A value is not known; however, it is known that animals depleted for a 28-day period exhibit symptoms of vitamin A deficiency, and it is not in accord with such observations

to find vitamin A liver stores of such magnitude in depleted rats.

In the third experiment designed to determine the cause of the increase in spectro vitamin A in the livers of the rats maintained on a vitamin A free diet for a period of 2 weeks, yeast was selected as the component of the diet most likely to be responsible. It was found upon spectrographic analysis that yeast did contain a substance capable of giving a false vitamin A value. Other substances were found by Fraps and Kemmerer ('38) to give false vitamin A values, and such substances are referred to as pseudo vitamin A.

Data obtained from the third experiment are presented in table 4. Those rats receiving diet 678-B which had the yeast

TABLE 4

Effect of diet low in vitamin A potency upon spectro vitamin A in rat livers.

	NO. OF RATS	SPECTRO VITAMIN A PER LIVER, IN MICROGRAMS		
		Range	Mean	Standard deviation of mean
Content at beginning	16	3.1– 8.5	5.8	1.6
Basal diet without yeast	8	8.4–13.3	10.5	2.0
Basal diet including yeast	8	9.9–19.4	15.4	3.5
Starch ration	8	10.9–15.1	13.4	1.9

Differences between litters is significant; difference between diet groups is significant.

replaced with cornstarch showed the least increase of pseudo vitamin A in the liver. The rats in the group receiving diet 7027-A which contained a small percentage of yeast had a significantly greater amount of pseudo vitamin A, while the group of rats receiving the regular basal diet (678-B) which is vitamin A free but contains the greatest amount of yeast, showed the greatest storage of pseudo vitamin A. All differences between diet groups are significant, and it is quite evident that the yeast was responsible for a considerable portion of the increase in the liver stores of pseudo vitamin A. Further examination would probably show that other com-

ponents of the diet were also contributing to the increase of the liver stores. It is obvious that equalized food intake is necessary in all cases where direct comparisons are made of the relative availability of different sources of vitamin A potency using the liver stores of vitamin A as a basis for the evaluation.

SUMMARY AND CONCLUSIONS

Using paired feeding technique and spectrographic analytical methods, investigations have been made on the utilization of vitamin A and carotene for liver storage of this vitamin in rats.

Vitamin A in cod liver oil was found to be most efficient for building up liver stores, carotene dissolved in cottonseed oil was 59% as effective, and carotene as found in alfalfa leaf meal was 21% as effective.

Carotene in alfalfa leaf meal was found to be 35% as efficient as carotene dissolved in cottonseed oil for building up the rat's liver store of vitamin A.

The spectro vitamin A content of the liver was found to increase significantly during the 2 weeks following weaning despite the fact that the experimental rats received a vitamin A free diet. No further significant increase occurred in the livers of litter mates that were continued on the same diet for an additional 2 weeks. Yeast present in the diet was found to contribute significantly to this increase.

The importance of equalizing food intakes and using litter mates in experiments involving the relative utilization of vitamin A and carotene, is stressed particularly when chemical and physical quantitative analytical procedures are employed in conjunction with the animal experimentation.

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A LIVER CONCENTRATE AS A SOURCE OF UNRECOGNIZED VITAMINS REQUIRED BY THE CHICK[1]

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ONE FIGURE

(Received for publication February 16, 1942)

It was shown by Hogan and Boucher ('33) that liver extracts are an excellent source of the unrecognized vitamins required by the chick. Simplified diets which contain these extracts promote a rapid rate of growth and prevent abnormalities often encountered in chicks on this type of ration. According to Hogan and Richardson ('40) three fractions of dried beef liver were required for the maximum rate of growth. These were (1) a 95% alcohol extract, (2) a water extract which followed the alcohol extraction, and (3) either the residue which followed the alcohol and water extractions or the acid-hydrolyzed residue. Although the separation was not complete, the evidence seemed to indicate that each fraction contained at least one essential nutrient which was more concentrated in it than in the other two.

Many new vitamins are now available in pure form, and it is possible to use less complicated rations than those formerly employed. The object of this report is to describe the degree of success obtained in substituting synthetic vitamins for the crude vitamin carriers formerly employed.

[1] Contribution from the Missouri Agricultural Experiment Station Journal Series No. 808.

EXPERIMENTAL

The experimental procedure was the same as that previously described (Hogan and Richardson, '40). The chicks were approximately 1 day old when delivered from the hatchery, and they were supplied with the experimental ration immediately. The composition of the basal ration, which is similar to those described in earlier publications, is given in table 1.

TABLE 1

Composition of basal ration.

Casein	35
Starch	31
Lard	17
Salts (Hubbell et al. ['37] with $MnSO_4$ added to make 0.025 Mn in the ration)	4
Cellulose	3
Gelatin	10
Vitamins[1] per 100 gm. of ration	
Vitamin A percomorph liver oil	6000 I. U.
Vitamin D	850 I. U.
Thiamine[2]	0.8 mg.
Riboflavin[2]	1.6 mg.
Pyridoxine[2]	1.2 mg.
Calcium pantothenate[2]	2.0 mg.
Choline[2]	400.0 mg.
Inositol	100.0 mg.
p-aminobenzoic acid	30.0 mg.
Nicotinic acid[2]	1.0 mg.
2 methyl-1,4-naphthoquinone[2]	1.0 mg.
Alpha tocopherol[2]	8.0 mg.
Biotin[3]	2.0 μg. per chick per day

The crude vitamin carriers were substituted on a dry matter basis for an equal weight of starch.

[1] This ration contains large quantities of vitamins and in some instances it may contain more than is required, but there was never any indication of toxicity. In general the results were more uniform when the vitamins were supplied in large quantity than when they were supplied in smaller amounts. The vitamins were added to the ration as solutions during the process of mixing to insure even distribution.

[2] Generously supplied by Merck and Co., Rahway, New Jersey.

[3] Concentrate no. 200, S. M. A. Corporation, Chicago, Illinois.

Preparation of the water extract of liver. In the earlier experiments the liver fractions were prepared by the method previously described by Hogan, Richardson, Patrick and Kempster ('41). It is now possible to eliminate both the alcohol extract and the acid-hydrolyzed liver residue. The water extract which is still indispensable may be prepared by a simpler method. In this new procedure fresh beef liver is extracted with boiling water. The water extract is concentrated to a convenient volume, and a large portion of the inactive material is removed by the addition of 95% alcohol to yield a concentration of 50%. This extract is concentrated to a thick paste and is designated the water extract of liver (WE). It contains all the unrecognized vitamins required by the chick, unless some unsuspected ones are concealed in the other organic constituents of the diet. The active agent is adsorbed from the water extract on fuller's earth at a pH of 1.0 (FEA), and is then eluted from the adsorbate with 0.2 N ammonia (FEE). A condensed description of these procedures is given in table 2.

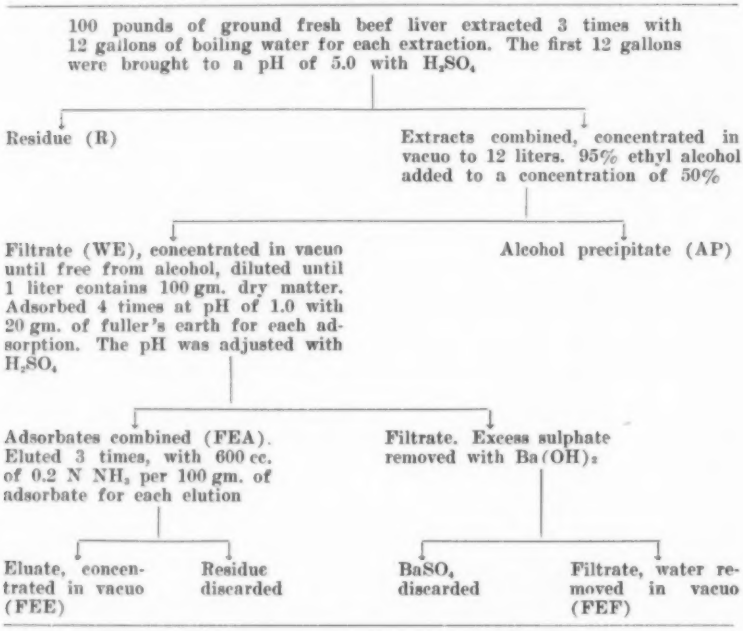
The alcohol extract is dispensable. Since it was stated in earlier reports from this laboratory that the 95% alcohol extract is indispensable, our present view that it is not essential needs some explanation. This fraction supplied pyridoxine and pantothenic acid, both of which are required by the chick and are now supplied in the ration in the pure form. In addition to these two vitamins this fraction supplied a specific organic antiperotic substance (Hogan, Richardson and Patrick, '40) which has been identified as choline (Jukes, '40 a, '40 b; Hogan et al., '41; Hegsted, Mills, Elvehjem and Hart, '41). The alcohol extract, therefore, is not essential when the ration contains 0.1% of choline in addition to the other vitamins which are now available in pure form. Larger amounts of choline do not improve the ration.

Gelatin may be substituted for the residue fraction. In view of the reports that glycine and other compounds are essential for the growth of chicks (Almquist and Mecchi, '40;

Almquist, Stokstad, Mecchi and Manning, '40 a, '40 b; Stokstad, Almquist, Mecchi, Manning and Rogers, '41; and Hegsted, Briggs, Elvehjem and Hart, '41), gelatin was substituted for the acid-hydrolyzed liver residue. When all the recognized vitamins were supplied, the rate of growth was as rapid with gelatin as with the hydrolyzed material. There

TABLE 2

Scheme followed in preparation of various fractions.


100 pounds of ground fresh beef liver extracted 3 times with 12 gallons of boiling water for each extraction. The first 12 gallons were brought to a pH of 5.0 with H_2SO_4
Residue (R)
Extracts combined, concentrated in vacuo to 12 liters. 95% ethyl alcohol added to a concentration of 50%
Filtrate (WE), concentrated in vacuo until free from alcohol, diluted until 1 liter contains 100 gm. dry matter. Adsorbed 4 times at pH of 1.0 with 20 gm. of fuller's earth for each adsorption. The pH was adjusted with H_2SO_4
Alcohol precipitate (AP)
Adsorbates combined (FEA). Eluted 3 times, with 600 cc. of 0.2 N NH_3 per 100 gm. of adsorbate for each elution
Filtrate. Excess sulphate removed with $Ba(OH)_2$
Eluate, concentrated in vacuo (FEE)
Residue discarded
$BaSO_4$ discarded
Filtrate, water removed in vacuo (FEF)

is no certainty that gelatin is free from contamination with vitamins and it would be preferable to omit it, but at present it is decidedly useful. It is probably preferable to the acid-hydrolyzed residue, as it is presumably less complex.

Unrecognized vitamins of the water extract of liver. In our earlier studies all attempts to dispense with a water extract of liver, such as preparation 4080, had ended in complete

failure, but at that time only a small proportion of the vitamins now recognized had been isolated, and were available. It seemed desirable, therefore, to reinvestigate the nutritional properties of the water extract to determine whether or not this fraction is still indispensable. The data are summarized in figure 1. Group IV received the basal diet and grew very slowly. In addition this group developed a perosis that is not due to a deficiency of either manganese or choline (Richardson and Hogan, '41). Group III received a fuller's earth eluate of the water extract, but biotin was omitted from the vitamin mixture. The growth rate was improved, but every chick developed dermatitis on the feet as described by Hegsted, Oleson, Mills, Elvehjem and Hart ('40). Group II received both the eluate and biotin. The growth rate was normal and dermatitis did not develop. Group I received the water extract itself, but the chicks did not grow any more rapidly than did those in group II. Since the vitamin mixture of group I did not contain biotin it is clear that this vitamin is present in the water extract.

The data in figure 1 show that the water extract of liver contains nutrients, in addition to the recognized vitamins, that are required for normal development of the chick. About 60% of the total activity of the original water extract was recovered in the fuller's earth eluate, and on the basis of equivalent amounts of dry matter, the active agent is concentrated approximately tenfold by this procedure. The remainder of the activity was lost, apparently due to destruction while in contact with acid during the adsorption process. Attempts to reduce this loss by adsorption in less acid solutions did not improve the procedure, because the amount of the active agent that was adsorbed decreased as the pH of the solution was increased. Only a small amount was adsorbed at a pH of 5.5. Our data indicate that the eluate is fully equal to the original water extract as a source of the unrecognized vitamins required by the chick.

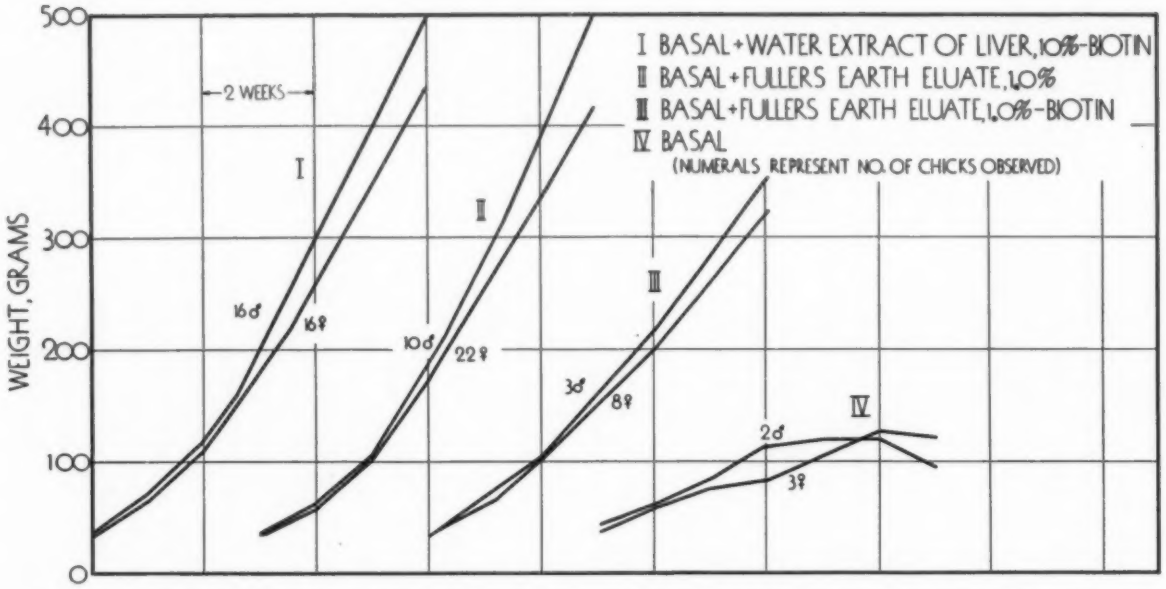


Fig. 1 The rate of growth on the basal ration (IV) is unsatisfactory, and most of the chicks developed perosis. When the fuller's earth eluate is added to the basal diet (II) the growth rate is normal, but when biotin is omitted from the vitamin mixture (III) dermatitis develops and the growth rate is retarded. Biotin may be omitted when the water extract (I) is supplied. The water extract evidently supplies this vitamin.

SUMMARY

1. When chicks receive a ration that contains no vitamins except those now recognized as such, they grow slowly and develop perosis.

2. A water extract of beef liver contains all unrecognized vitamins required by the chick.

3. The unrecognized vitamins required by chicks are adsorbed from a water extract of liver by fuller's earth at a pH of 1.0. The activity is readily eluted by 0.2 N ammonia. During the adsorption procedure approximately one-third of the total activity is lost.

4. A simplified diet has been prepared that is adequate for the growth of chicks and does not contain over 1% of crude vitamin carriers.

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POLYNEUROPATHY IN THIAMINE DEFICIENT RATS DELAYED BY ALCOHOL OR WHISKY [1]

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FOUR FIGURES

(Received for publication March 6, 1942)

INTRODUCTION

In 1934 Cowgill proposed a formula relating the vitamin B requirement to body weight and caloric intake. This relationship was used by Jolliffe and co-workers (Jolliffe, Colbert and Joffe, '36; Jolliffe and Colbert, '36) in their calculations to show that a deficiency of vitamin B (B_1) was present in the diets of alcoholic, polyneuritic patients. The occurrence of polyneuritis could not be predicted consistently unless the calories from alcohol were included with the diet calories. Laboratory investigations (Evans and Lepkovsky, '29, '34, '35; Kemmerer and Steenbock, '33; McHenry, '37; Salmon and Goodman, '37) have shown that the vitamin B_1 requirement is influenced by the composition of the diet as well as by the total number of calories. An abundant carbohydrate intake increases the need for thiamine while the substitution of fat for carbohydrate lessens the need and delays the onset of polyneuropathy. Since there was no evidence to the contrary, the assumption has been retained that the metabolism of alcohol increased the need for thiamine, and on this basis alcohol has been indicted as a more or less specific factor in producing the polyneuropathy seen in alcoholic patients.

[1] Read at the Boston meeting of the American Institute of Nutrition, April 1, 1942.

Because of the lack of direct evidence on the relationship of alcohol intake to the thiamine need, an experimental investigation was undertaken.

EXPERIMENTAL

Albino rats at weaning (22 days old) were used throughout the experiment; paired rats were litter mates of the same sex. The rats were housed in individual, elevated, wide-mesh galvanized wire cages. Two strains of rats were used, one in the experiments with whisky and another in the alcohol experiments. The ethyl alcohol used was approximately 20% by volume and the whisky was a 100 proof commercial brand diluted with an equal volume of water.

All the animals were fed diet no. 461 which had the following percentage composition: leached and alcohol extracted casein, 18; sucrose, 73; cottonseed oil,[2] 3; cod liver oil, 2; Osborne and Mendel salt mixture, 4. A daily supplement of 50 μg. of riboflavin, 50 μg. of calcium pantothenate, 20 μg. of pyridoxine, 1 mg. of nicotinic acid, and 2 mg. of choline was given to each rat in a supplement dish. For the first 42 days 4 μg. of thiamine chloride were added to the daily vitamin supplement. The number of days from the complete withdrawal of the thiamine to the onset of acute polyneuropathy was recorded. Polyneuropathy was considered to be present when the rat was spastic, and had an ataxic, tonic convulsive seizure when dropped on its back from a height of 3 to 5 inches. Death occurred in untreated rats within 48 hours of the time that this syndrome was first observed.

Experiment I

Effect of whisky with isocaloric intake

Twelve pairs of litter mates were used for this experiment. One rat in each pair drank whisky ad libitum, and the other, water ad libitum. The two rats of each pair received an

[2] Wesson.

isocaloric intake throughout the experiment (based on 4.1 calories per gram for the food and 1.5 calories per cubic centimeter for the diluted whisky).

The whisky intake was recorded by means of an especially designed apparatus which collected the whisky wasted by the animal. A 10% allowance was made for evaporation based on losses from a similar apparatus set up in an adjoining empty cage. The apparatus was filled with a measured quantity of whisky. The collected waste and the remainder were measured and the dietary caloric equivalent of the consumed whisky was calculated. The amount of food offered to the two rats of each pair was adjusted daily to equalize the caloric intake according to the calculated average daily intake of whisky. After the development of polyneuropathy in either of the paired rats, the remaining animal was fed diet no. 461 ad libitum.

Because of the calories supplied by the whisky the average intake of diet no. 461 for the rats receiving whisky was 74 to 82% (average 79%) of the amount of this diet eaten by the rats getting water. The average daily intake of diet no. 461 up to the time of development of polyneuropathy (a) of the rats receiving whisky was 3.9 gm., and (b) of the rats receiving water, 5.0 gm.

Results. In every one of the twelve pairs of rats polyneuropathy first occurred in the rat that drank water (fig. 1). In the group as a whole the average time between the withdrawal of thiamine chloride and the onset of polyneuropathy in the rats on water was 18 days; in the rats on whisky, 33 days (an 83% longer interval). During the 42-day preliminary period in eleven of twelve pairs the rats receiving whisky had a gain in body weight which was less (average 23%) than that of the litter mates receiving water. In one pair the rat that drank water gained less (9%) than the rat receiving whisky. A rapid decline in body weight occurred in both the rats getting whisky and those receiving water following the complete withdrawal of the thiamine.

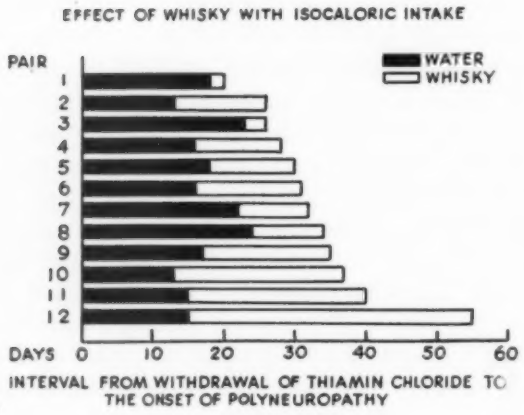

Figure 1

Experiment II

Effect of whisky with equal food intake

Twelve pairs of litter mates were used for this experiment. The rats of each pair were allowed equal amounts of diet no. 461 until one developed polyneuropathy. The paired rat was then fed diet no. 461 ad libitum. Throughout the experiment one rat in each pair was given diluted whisky instead of drinking water. The whisky gave these rats, on the average, a 28% higher caloric intake than that of the rats that drank water. The average daily intake of diluted whisky was 3.7 cc.

Results. In eleven of the twelve pairs polyneuropathy first occurred in the rat drinking water (fig. 2). The twelfth pair was discarded because one of the rats (receiving whisky) died without observed polyneuropathy. There were no abnormal post mortem findings except emaciation. In the eleven pairs the average time between the withdrawal of thiamine chloride and the onset of polyneuropathy in the rats on water was 19 days; in the rats on whisky, 29 days (a 53% longer interval). The rat on water limited the diet intake in every pair.

During the 42-day preliminary period the rats on whisky in seven of the eleven pairs had a greater gain in body weight (average 11%) than the litter mates on water. In one pair the weight gain was equal. In three pairs the rats on water had a greater gain of weight (average 13%) than the litter mates on whisky. Following the withdrawal of thiamine a rapid decline in weight occurred in both the rats receiving water and those getting whisky.

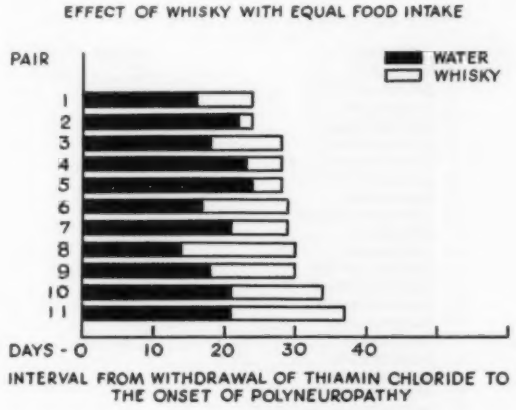

Figure 2

Experiment III

Effect of ethyl alcohol with equal food intake

This experiment is similar to experiment II. Throughout the experiment one rat in each pair was given alcohol ad libitum (approximately 20% by volume) instead of drinking water. The amount of alcohol taken was not recorded. Twenty-four pairs of litter mates were used. In each pair, the rats were fed an equal amount of diet no. 461 up to the time of development of polyneuropathy in one of the rats of the pair; the paired rat was then fed diet no. 461 ad libitum.

Results. In twenty-three of the twenty-four pairs, polyneuropathy first occurred in the rat on water (fig. 3). The

twenty-fourth pair was discarded because one of the rats (that got water) died without observed polyneuropathy and post mortem examination showed pneumonia. In the twenty-three pairs the average time between the withdrawal of thiamine chloride and the onset of polyneuropathy in the rats on water was 14 days; in the rats on alcohol, 22 days (a 57% longer interval). The rat on water limited the diet intake in every pair.

Figure 3

During the 42-day preliminary period the rats getting alcohol in nineteen of twenty-three pairs had a gain of weight that was greater (average 23%) than that of the litter mates receiving water. In one pair the gain was equal, and in three pairs the rats that drank water had a greater gain (average 13%) than the litter mates that received alcohol. After the withdrawal of the thiamine there was a rapid decline in body weight in both the rats getting alcohol and those drinking water.

Experiment IV

Effect of food restriction with ethyl alcohol

Twelve litters of three rats each were fed diet no. 461. In each litter, rat A was given drinking water and diet no. 461 ad libitum. Rat B was given alcohol and received the same amount of diet no. 461 as rat A until the thiamine was withdrawn, and was then allowed to eat ad libitum. Rat C was given alcohol and had the intake of diet no. 461 restricted to

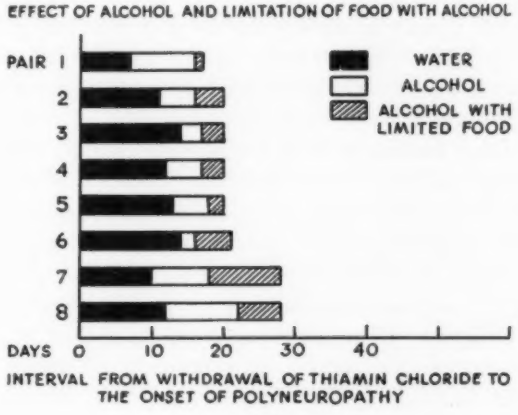

Figure 4

80% of the amount eaten by rat B. At the time of development of polyneuropathy in rat B, rat C was fed diet no. 461 ad libitum.

Results. In eight of the twelve litters polyneuropathy occurred first in rat A (on water), next in rat B (on alcohol) and last in rat C (on alcohol with a restricted diet intake) (fig. 4). In the remaining four litters polyneuropathy was not observed prior to death in at least one rat in each litter.

In the eight litters in which polyneuropathy was observed in all of the rats the average time from the withdrawal of thiamine chloride to the onset of the polyneuropathy in the rats on water (A) was 12 days; in the rats on alcohol (B)

18 days (a 50% longer interval) and in the rats on alcohol with a restricted food intake (C) 22 days (an 83% longer interval). The rat on water (A) limited the diet intake in every litter. The rats on water (A) had an abrupt decrease in the intake of food after the withdrawal of thiamine. The litter mates on alcohol (B) that had the same intake of diet no. 461 until the withdrawal of thiamine had a more gradual decrease in the amount of diet eaten daily. The rats on alcohol (B) consumed 26% more of diet no. 461 than the rats receiving water (A) from the time of withdrawal of the thiamine to the onset of polyneuropathy in the rat getting water (A). During this period the average daily diet consumption of the rats on water (A) was 1.9 gm., and of the rats on alcohol (B) 2.5 gm.

In the four litters in which polyneuropathy was not observed in at least one rat in each litter, one rat on alcohol (B) and three on alcohol with a restricted food intake (C) died without showing evidence of polyneuropathy. No pathological changes except emaciation were found at the post mortem examination. All four of the rats on water (A) died without showing evidence of polyneuropathy, and post mortem examination revealed a large accumulation of clear, colorless fluid in the thoracic and peritoneal cavities. The hearts appeared enlarged and both ventricles were filled with blood. The histological examination of the hearts of these four animals showed short or long areas of the auricular myocardium in which muscle fibers were absent or decreased in number with a consequent marked thinning of the wall in three animals. In such areas there was slight to moderate fibroblast proliferation, slight collagen deposition, and occasional to few lymphocytes. The auricle of the fourth heart was not present in the section examined. These lesions were not present in the ventricular myocardium.

Experiment V

Prevention and treatment of polyneuropathy by thiamine

Three litters of four rats each were fed diet no. 461. Two rats in each litter were given diluted whisky, and two were

given water. The four litter mates received an isocaloric intake based on the caloric value of the amount of diet no. 461 eaten plus the whisky. In each litter one rat on whisky and one rat on water were given 15 μg. of thiamine chloride daily by mouth added to the supplement of the other vitamins; one rat on whisky and one rat on water were on the thiamine deficient regime. (The rats on the thiamine deficient regime are the same animals reported under litters no. 6, 10 and 11 in experiment I). Twenty-five rats from experiments I–IV were given 200–400 μg. of thiamine chloride immediately after the first appearance of polyneuropathy.

Results. None of the rats on water or whisky that received 15 μg. of thiamine chloride daily showed any signs of polyneuropathy. The litter mates on the thiamine deficient regime getting either water or whisky developed polyneuropathy as shown in table 1. (Several hundred rats receiving 20% ethyl

TABLE 1

Days required for development of polyneuropathy.

LITTER	B_1 DEFICIENT REGIME AND RECEIVING		B_1 DEFICIENT REGIME + 15 μG. B_1 DAILY AND RECEIVING	
	Water	Whisky	Water	Whisky
6	16	31	No polyneuritis	No polyneuritis
10	13	37	No polyneuritis	No polyneuritis
11	15	40	No polyneuritis	No polyneuritis

alcohol as a source of fluid and eating various diets containing adequate thiamine have been maintained in this laboratory for periods as long as 1 year without developing polyneuropathy.) In the twenty-five rats treated with thiamine chloride immediately after the onset of polyneuropathy, the symptoms disappeared within 24 hours whether the animal was on alcohol, whisky, or water.

DISCUSSION

In paired feeding experiments (I–IV above) with a thiamine deficient diet, fifty-four rats on water without exception developed polyneuropathy before the paired litter mates on

alcohol or whisky. It is clear from the results of the above experiments that under these conditions alcohol and whisky caused a delay in the onset of the polyneuropathy. No definite explanation of the mechanism of this delay is evident. It is obvious that the data do not support the assumption that the ingestion of alcohol increases the thiamine requirement.

The results of the preventive and therapeutic experiment (V) demonstrate that the polyneuropathy observed was related to the thiamine deficiency and could be prevented or alleviated by the administration of thiamine regardless of the presence or absence of alcohol or whisky. It is interesting to note that the four rats on water in experiment IV that died without having polyneuropathy that was observed showed hydrothorax, ascites, and histopathological changes in the heart. Drury, Harris and Maudsley ('30) observed bradycardia, and Weiss, Haynes and Zoll ('38) have described electrocardiographic changes in thiamine deficient rats.

CONCLUSION

Under the conditions of these experiments the ingestion of alcohol or whisky delays the onset of polyneuropathy in thiamine deficient rats.

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THE THIAMINE AND RIBOFLAVIN CONTENTS OF CITRUS FRUITS [1]

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(Received for publication February 21, 1942)

Owing to the paucity of data concerning the thiamine and riboflavin contents of citrus fruit, the work reported herein was undertaken.

THIAMINE

The comprehensive review by Daniel and Munsell ('37) gives the following values (rat growth assays) for grapefruit and oranges — 20 and 60 Sherman-Chase units per 100 gm., respectively. By use of the rat bradycardia method, the value of 0.4 International Unit (equal to 1.2 μg.) per gram has been found for grapefruit, oranges and tangerines (Baker and Wright, '35). In a recent rat growth study, grapefruit and oranges (Florida) have been reported (Booher and Hartzler, '39) to contain 72 and 78 μg. per 100 gm., respectively. The value of 111 μg. per 100 gm. for oranges has been reported by Pyke ('39), the method of analysis involving fluorophotometry.

The authors of this paper have analyzed specimens of Florida grapefruit and oranges purchased in New York during the season of November, 1939, through May, 1940, and Florida tangerines purchased in December, January and February of the same season.

[1] Read on September 11, 1941, before the Division of Agricultural and Food Chemistry at the 102nd meeting of the American Chemical Society at Atlantic City, N. J. Published by permission of the American Chemical Society.

In the case of each sample taken, a standard box (1.6 bushel capacity, grapefruit and oranges, and 0.8 bushel for tangerines) was purchased and one-fifth of the number of fruit in a box was taken for analysis. Thus one sample may have been derived from a number of different trees.

The juice was expressed by a pressure, not by a reaming method. This mixed juice was then analyzed for its thiamine content. The method of analysis was in principle that of Hennessy and Cerecedo ('39) with certain modifications as suggested by Dr. W. L. Sampson of the Merck Institute. In short, the thiamine was adsorbed on permutit, eluted with acidified potassium chloride solution and oxidized to thiochrome by means of alkaline ferricyanide solution. The thiochrome was extracted from the alkaline aqueous solution by isobutanol and the intensity of its fluorescence measured in a Pfaltz and Bauer fluorophotometer, with prescribed "blank" correction.

The expressed juice was, prior to analysis, stored in stoppered bottles at 2°–5°C. for 12–24 hours to effect a sedimentation of the pulp. The supernatant pulp-free, although cloudy, juice was taken for analysis. It was adjusted to pH = 4 to 4.5, takadiastase added, incubated from 1 to 1.5 hours at 37–38°C., heated to boiling and cooled to 80°C. before being passed through the permutit column. It was found that the enzyme treatment was essential and that it made no difference whether the supernatant pulp-free juice or juice containing pulp was incubated with the enzyme. Hence, owing to the greater convenience, the procedure as mentioned was adopted.

The results are given in table 1. The size designations are the number of individual fruits which just fill the standard Florida box — 1.6 bushel for grapefruit and oranges, and 0.8 bushel for tangerines. Based on the average juice volume of these particular specimens the thiamine chloride hydrochloride or vitamin B_1 expressed in micrograms per fruit of the size indicated is 80 per pineapple orange, 91 per Valencia orange, 85 per seeded grapefruit, 81 per seedless grapefruit and 45 per tangerine.

TABLE 1

Vitamin B_1 content of certain citrus fruits.

FRUIT	SIZE	NUMBER OF BOXES SAMPLED	TOTAL NUMBER OF FRUITS TAKEN	VITAMIN B_1 [1] MICROGRAMS PER 100 ML. JUICE			
				Highest	Lowest	Mean	S. D.[2]
Oranges							
Pineapple	176	11 [3]	385	76	54	65	6.1
Valencia	176	11	385	81	58	70	6.7
Grapefruit							
Seeded	70	9	126	47	18	35	8.6
Seedless	70	10	140	43	18	32	7.5
Tangerines	150	5	150	78	59	69	6.4

[1] Calculated as thiamine chloride hydrochloride.

[2] Standard deviation from the mean.

[3] Including one box of Parson Brown oranges, analysis of which yielded 63 μg. of vitamin B_1 per 100 ml. juice.

RIBOFLAVIN

The literature gives a wide divergence in values for the riboflavin content of citrus fruit based on assays of but a few specimens. In the Daniel and Munsell ('37) survey, the values given for grapefruit and oranges are 40 and 35 Sherman-Bourquin units per 100 gm., respectively. These figures are, in the opinion of the present authors, too high (assuming one Sherman-Bourquin unit to be equal to about 2.5 μg. of riboflavin). On the other hand, the low values of 6.9 and 8.9 μg. per 100 ml. of orange juice have been reported (Kuhn, Wagner-Jauregg and Kaltschmitt, '34). These values are undoubtedly much too low owing to the fact that they were obtained by the lumiflavin method in 1934.

Recently by rat growth assay (Lanford, Finkelstein and Sherman, '41), the values of 20.0 and 27.8 μg. per 100 gm. have been reported for grapefruit and oranges, respectively, and in India (Wilson and Roy, '38) the contents of 24 μg. (grapefruit) and 59 μg. (orange) per 100 gm. have been published.

In the present study, the riboflavin content of representative specimens of oranges and of grapefruit was measured fluoro-

metrically by a simple direct method described below. Each lot of citrus fruit was purchased in New York City on the day it arrived from Florida through the period December 16, 1940, to May 12, 1941. As in the case of the thiamine measurements, the juice of one-fifth of the total number of grapefruit or oranges in a box was expressed and this mixed juice was taken as a sample.

The mixed expressed juice was stored in darkness at 2°–5°C. for 12 to 24 hours in completely filled glass bottles and then filtered through paper. This storage preliminary to filtration results in a flocculation of colloidal matter and greatly facilitates the production of a clear filtrate. Clarity is essential, because even a slight haze greatly increases the degree of quenching of the fluorescence. The first 50–75 ml. of filtrate were discarded and the filtration continued in a refrigerator at 2°–5°C., and, of course, in absence of light.

The intensity of fluorescence was measured by use of a fluorophotometer,[2] the incident light passing through a combination of Corning filters nos. 038 and 511. Between the cuvette and photronic cell there was placed a Corning filter no. 351.

When the Klett instrument was used, the opposing photronic cell was incited by the fluorescence of a quinine sulfate solution (0.15 mg. quinine sulfate per liter of tenth normal sulfuric acid solution). The incident light was filtered through a Corning no. 584 filter and the combination of filters nos. 038 and 428 was placed between the quinine solution and photronic cell.

The fluorescence of the following solutions was measured: (A) 25 ml. of filtered juice diluted to 50 ml. with distilled water; (B) 25 ml. of filtered juice diluted to 50 ml. with a standard riboflavin solution producing a riboflavin increment of 0.05 μg. per milliliter; (C) same as B except that riboflavin increment was 0.075 μg. per milliliter; (D) any two of A, B and C treated with hydrosulfite for "blank" correction.

[2] Both the Pfaltz and Bauer and the Klett instruments were used.

The hydrosulfite solution (Sullivan and Norris, '39) was prepared by dissolving 5 gm. of sodium hydrosulfite in 100 ml. of an ice-cold sodium bicarbonate solution (2 gm. $NaHCO_3$ per 100 ml.). This solution was added to A, B or C in the proportion of 3 ml. of the former to 25 ml. of the latter.

A typical calculation may be helpful: The photometer scale readings of these solutions in one experiment were — A = 121, B = 179, C = 210 and D = 34. Hence, the readings corrected for the "blank" were — A = 87, B = 145, C = 176 and

$$\frac{0.05}{145-87} \times 87 = 0.0750; \quad \frac{0.075}{176-87} \times 87 = 0.0733$$

Thus the indicated riboflavin content of A is 0.074 μg. per milliliter and that of the filtered juice is 0.15 μg. per milliliter.

This method renders unnecessary the preparation of a standard of reference curve of photometer response plotted against riboflavin concentration unless one desires to know the extent to which the intensity of fluorescence of riboflavin is quenched by substances in the solution analyzed. In the example cited the degree of quenching was thus found to be 35%. Obviously, either solution B or C is sufficient for an analysis. The authors preferred, however, to run both B and C as "checks" at two levels of added riboflavin. All analytical operations were performed in a dimly lighted room.

The results by this method were compared with those obtained by use of the Snell and Strong ('39) microbiological method in three instances.

Specimens of filtered grapefruit juice (our specimen no. 634) and of filtered orange juice (our specimens no. 639 and 642) yielded the results of 14, 18 and 15 μg. of riboflavin per 100 ml., respectively, by the method described in this paper and 14, 18 and 14 μg., respectively, by the Snell and Strong method.[3]

Higher results were obtained, however, when the microbiological method was applied to unfiltered juice. The values of 22 and 18 μg. per 100 ml. were obtained for unfiltered specimens 639 and 642, respectively. Since these higher results (if

[3] The microbiological assays were kindly performed by Mr. Carol E. Weill.

valid) indicated that riboflavin might be either bound to constituents of the cellular structure of the pulp or adsorbed by the filter paper, further experiments were performed. It was found that the prescribed conditions of filtration were not at fault.

Several extractive procedures were applied to the unfiltered juice. A specimen of orange juice was heated by immersion in boiling water for 1.5 hours under a reflux condenser. It was cooled and filtered. The filtrate was darker in color than the original juice and gave a lower value for riboflavin. The fluorescence intensity after addition of hydrosulfite had increased also.

Since Hodson and Norris ('39) recommend extraction of solid samples with 0.25 N sulfuric acid, a specimen of unfiltered orange juice was rendered 0.25 N with respect to sulfuric acid and heated in boiling water for 25 minutes. Upon cooling, it was brought to pH = 5.53 by addition of saturated trisodium phosphate solution, diluted to a definite volume with distilled water and filtered. The filtrate gave within the error of the method the same riboflavin result as the untreated juice.

Acetone has been recommended as an extractant for milk by Hand ('39) who states also that at an acetone concentration of 66%, riboflavin combined with protein is disassociated therefrom. It was therefore considered of interest to try the effect of acetone upon citrus juice. Orange juice (unfiltered) and acetone were mixed at room temperature in the proportions of one volume of the former to two volumes of the latter. The filtrate was yellowish brown in color and the indicated riboflavin content of the juice was much lower than that of the untreated, filtered juice. Another result of the acetone treatment was the high fluorescence quenching power of the acetone soluble matter—the fluorescence intensity was quenched 85%; hence, no quantitative calculations would be acceptable.

Refluxing equal volumes of methanol and unfiltered orange juice for 25 minutes followed by cooling and filtering yielded a very satisfactory filtrate in which the fluorescence was quenched to the extent of only 26%. The riboflavin content

found by this method was the same as by direct measurement of untreated filtered juice.

The riboflavin content of certain citrus fruits as determined by the simplified method described herein is given in table 2.

TABLE 2

Riboflavin content of certain citrus fruits.

FRUIT	SIZE	NUMBER OF BOXES SAMPLED	TOTAL NUMBER OF FRUITS TAKEN	RIBOFLAVIN MICROGRAMS PER 100 ML. JUICE			
				Highest	Lowest	Mean	S. D.[1]
Oranges							
Pineapple	176	7	245	17	13	16	1.3
Valencia	176	7	245	18	15	15	1.1
Grapefruit							
Seeded	70	8	112	14	10	12	1.4
Seedless	70	8	112	12	9	11	1.1

[1] Standard deviation from the mean.

Based on the average juice volume of these particular specimens, the riboflavin per fruit (of the size designated) is 20 μg. per orange, and 27 μg. per grapefruit.

ACKNOWLEDGMENT

Grants-in-aid in this investigation made by the Florida Citrus Commission are gratefully acknowledged.

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STORAGE OF PYRIDOXINE IN THE RAT [1]

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(Received for publication February 28, 1942)

Although it is generally assumed that animals have a limited capacity for storing the various factors of the vitamin B complex, little evidence has been presented to demonstrate this fact. We have observed that weanling rats on pyridoxine-free rations require a much longer period to develop dermatitis when they are allowed to continue feeding on the stock diet and thus increase their weight over that of controls. This suggested a capacity for storing pyridoxine. Such capacity was demonstrated when graded doses of this factor were administered over an initial 10-day period to rats maintained on the pyridoxine-free diet. The more vitamin the rats obtained during this period, the longer were they able to resist the onset of dermatitis. It is the object of this paper to present data which substantiate these findings.

EXPERIMENTAL

Rats of the Wistar strain, reared in our laboratory, were employed. The basal diet (diet A) had the following percentage composition: casein, vitamin free [2] 30, sucrose 55, hydrogenated cottonseed oil [3] 5, cod liver oil 3, Osborne and Mendel salt mixture 7. Supplements of 10 μg. of thiamine chloride, 20 μg. of riboflavin, and 0.3 cc. of a fuller's earth filtrate of pig liver extract [4] equivalent to 3 gm. of fresh liver were fed daily.

Normally, rats weighing about 40 gm. at weaning, when placed immediately on our pyridoxine-free ration, develop

[1] Presented at the meeting of the American Chemical Society at Detroit, September, 1940.

[2] Labco. [3] Crisco. [4] Prepared by the method of Lepkovsky et al. ('36).

dermatitis in about 1 month. However, one group of animals, allowed to continue on the stock diet [5] for 10 days after weaning before they were given the deficient diet, averaged 51 gm. of body weight and did not develop dermatitis until after 2½ months. This observation led us to select litters of varying ages and body weights and to compare their growth and the time of appearance of dermatitis on the pyridoxine-free diet. The results obtained are given in table 1. They show that with increasing initial body weight the incidence of dermatitis was correspondingly delayed.

TABLE 1

Effect of initial body weight on growth and the time of appearance of dermatitis.

NO. OF RATS	AVERAGE INITIAL BODY WEIGHT	AVERAGE TIME OF APPEARANCE OF DERMATITIS	AVERAGE GAIN IN WEIGHT OVER 40 DAYS
	gm.	*days*	*gm./day*
6	51	77	0.55
8	41	40	0.43
5	36	35	0.33
7	21	28	0.48

TABLE 2

The growth and time of appearance of dermatitis in young rats whose mothers were depleted during the lactation period.

NO. OF RATS	PORTION OF THE LACTATION PERIOD USED	INITIAL BODY WEIGHT	AVERAGE TIME OF APPEARANCE OF DERMATITIS	AVERAGE GROWTH OVER 30 DAYS
		gm.	*days*	*gm./day*
40	None	38	33	0.33
4	⅓	35	24	0.27
16	⅔	25	19	0.17

In order to limit storage of pyridoxine prior to weaning, three nursing rats and their progeny were given the pyridoxine-free diet during the latter part of the lactation period. As indicated in table 2, these animals were of lower body weight and developed dermatitis at an earlier age than did normally reared young.

In the preceding experiments it has been shown that the higher the initial body weight of the rats when the animals are placed on the pyridoxine-free diet, the longer is the time

[5] Purina Dog Chow.

required for dermatitis to appear. It must be assumed that this increased body weight represents a proportional increase in the amount of pyridoxine accumulated in the tissues, and that the animal uses this factor sparingly. However, since there may be other factors that influence the appearance of dermatitis, this assumption is not entirely justified. Analysis of the body tissues for pyridoxine after increasing periods of time on the deficient ration would have furnished evidence of storage, but unfortunately the methods available at present for the estimation of pyridoxine are not sufficiently dependable. We, therefore, decided to select animals of the same body weight, give them graded doses of pyridoxine during a short initial period, and then observe whether the time of appearance of dermatitis could be related to the amount of the vitamin that they received.

A total of thirty-six rats were given diet A at weaning, and, after a 5, 10, or 15 day depletion period, were divided into four groups and given the pyridoxine-free diet (diet A plus the supplements). These four groups then received 0, 5, 10, and 15 μg., respectively, of pyridoxine over a 10-day period, after which this supplement was discontinued. The animals showed an increased growth rate and a delay in the appearance of dermatitis corresponding to the amount of pyridoxine initially fed (table 3).

This experiment was repeated with a group of twenty rats receiving diet A supplemented with 2 mg. of thiamine chloride, 4 mg. of riboflavin, and 20 mg. of d-calcium pantothenate [6] per kilo of diet. The animals were divided into four groups and given 0, 5, 10, and 15 μg., respectively, of pyridoxine daily for 10 days, after which time they were allowed to continue on the basal diet until dermatitis developed. The results, as shown in table 3, were the same as those obtained in the preceding experiment.

SUMMARY

The storage capacity for pyridoxine has been studied on young rats maintained on a vitamin B complex free diet

[6] The d-calcium pantothenate was generously supplied by Merck and Company, Rahway, N. J.

TABLE 3

The relation of growth and the time of appearance of dermatitis to the amount of pyridoxine ingested over an initial 10-day period.

NO. OF RATS	DEPLETION PERIOD	DAILY DOSE OF PYRIDOXINE	TIME OF APPEARANCE OF DERMATITIS	AVERAGE GAIN IN WEIGHT OVER 40 DAYS
	days	*μg.*	*days*	*gm./day*
4	5	15	59	1.3
4	5	5	43	0.9
4	5	0	28	0.5
4	10	15	58	0.9
4	10	5	45	0.55
4	10	0	30	0.2
4	15	15	70	0.9
4	15	10	60	0.8
4	15	0	35	0.47
5[1]	10	15	68	1.9
5[1]	10	10	56	1.75
5[1]	10	5	45	1.25
5[1]	10	0	29	0.95

[1] These animals were given pantothenic acid in place of liver filtrate.

supplemented with thiamine, riboflavin, and liver filtrate (or pantothenic acid).

If the rats are weanlings, they develop dermatitis in about 1 month; if they are allowed to subsist on the stock diet before being fed the pyridoxine-free ration, dermatitis does not develop until after 2½ months. If the mother is given the B_6-free diet during the latter part of the lactation period, the young grow more slowly and symptoms of B_6 deficiency appear earlier than otherwise. Depleted animals given stated amounts of pyridoxine over a 10-day period show a progressive increase in rate of growth and corresponding delay in onset of dermatitis correlated with the amount of the vitamin given.

These data suggest that the irregularity in the occurrence of dermatitis in rats deprived of pyridoxine (reported by several workers) may be ascribed, at least in part, to differences in the reserves of the vitamin present in the young animals at the beginning of the experiment.

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